AD_____

Award Number: DAMD17-99-1-9507

TITLE: c-Jun N-terminal Kinase and Apoptotic Signaling in Prostate Cancer

PRINCIPAL INVESTIGATOR: Yi-Rong Chen, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: July 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Annual Summary(1 Jul 99 - 30 Jun 00)

4. TITLE AND SUBTITLE
c-Jun N-terminal Kinase and Apoptotic Signaling in Prostate Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9507

6. AUTHOR(S)
Yi-Rong Chen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-MAIL:
yc691276@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Understanding the biochemical mechanisms of apoptosis is important for revealing cancer biology and for improving cancer therapies. The c-Jun N-terminal kinase (JNK) pathway participates in cellular responses to both environmental stresses and mitogenic signals. JNK is involved in cellular signaling during apoptosis induced by various agents including γ-radiation, UV-radiation, anti-carcinogenic isothiocyanates, and retinoid analog N-(4-hydroxyphenyl) retinamide (4-HPR) in various cancer cells. Interfering with the JNK pathway suppressed the induction of apoptosis. JNK activation by apoptotic stimuli can be caspase-dependent or independent. Different apoptotic stimuli induce JNK activation through distinct mechanisms. The involvement of JNK in both mitogenic and apoptotic signaling implies that a subtle regulatory mechanism must exist for the signaling decision. Our studies reveal that the duration of JNK induction may determine cell fate. In conclusion, our study reveals the importance of the JNK pathway in apoptotic signaling. These results provide important information for the studies of oncogenesis and the mechanisms of radiation and drug resistance in cancer cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Prostate cancer, JNK, apoptosis, signaling	44
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

URC Where copyrighted material is quoted, permission has been obtained to use such material.

URC Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

URC Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Yi-Rong Chen 07-28-00

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Apoptosis is cell death characterized by unique biochemical and morphological events including internucleosomal DNA cleavage, chromatin condensation, membrane blebbing, and cell fragmentation. Apoptosis plays an essential role in the development and maintenance of homeostasis in multicellular organisms. Apoptosis is positively and negatively regulated by many physiological and pathological factors, and this regulation is initiated by genetic and biochemical programs. Apoptosis occurs in normal physiological conditions, and can be induced by ionizing radiation, UV radiation, DNA-damaging drugs, oxidants, viral infection, and deprivation of growth factors. Disorders in apoptosis play critical roles in carcinogenesis and in the development of resistance to radiation or chemotherapy in cancer cells. Therefore, understanding the molecular mechanisms of the apoptotic process is important for the prevention and treatment of cancers.

JNK (c-Jun N-terminal kinase; also named as stress-activated protein kinase, SAPK) belongs to the mitogen-activated protein kinase (MAPK) superfamily, which also includes extracellular signal-regulated kinases (ERKs) and the p38-MAPK family. MAPKs are serine/threonine kinases that target other kinases, transcription factors, and membrane receptor tails, causing diverse effects such as cell proliferation, transformation, differentiation, and apoptosis. JNK family consists of three genes, JNK1, JNK2, and JNK3, which encode ten isoforms of proteins. Substrates for JNKs include the transcription factors c-Jun, JunD, ATF-2, ATFa, Elk-1, Sap-1a, and p53. Phosphorylation of c-Jun, ATF-2, Elk-1, and Sap-1a increases their transcriptional activity. JNK kinase activity is activated by pro-inflammatory cytokines (TNF-α and IL-1), G protein-coupled receptors, lymphocyte activation stimuli, osmotic shock, heat shock, protein synthesis inhibitors, ceramides, DNA-damaging chemicals, UV radiation, and γ radiation. Activation of JNK involves MKK4 (MAPK kinase 4, also called SEK1 [SAPK/ERK kinase] and JNKK1), MKK7 (JNKK2), and MEKK1 (MAPK kinase kinase 1). MEKK1 phosphorylates and activates the dual-specificity kinase MKK4/SEK or MKK7. MKK4/SEK (or MKK7) then activates JNK via threonine and tyrosine phosphorylation of the T-P-Y motif on JNK.

JNK is activated in apoptosis induced by growth factor withdrawal, UV-C, γ radiation, ceramide, heat shock, and DNA-damaging drugs. Activation of the JNK pathway leads to apoptosis. Interference with the JNK pathway by the dominant-negative mutant of MEKK1, MKK4/SEK, or JNK1 suppresses apoptosis. JNK's substrate, c-Jun, is required for ceramide-induced apoptosis and apoptosis of neuronal cells caused by NGF withdrawal. All these results indicate the importance of the JNK pathway in apoptosis. The JNK pathway participates in cellular responses to mitogens, stresses, and apoptotic agents. The mechanisms by which JNK integrates with other cellular signaling to achieve these diverse functions are intriguing. We have found that the induction of JNK in response to mitogenic and apoptotic signals have different activation patterns, transient versus persistent, respectively. Co-treatment of a tyrosine phosphatase inhibitor (sodium orthovanadate) and T-cell activation signals (phorbol 12-myristate 13-acetate [PMA] plus ionomycin) prolongs the JNK induction by T-cell activation agents and results in T-cell apoptosis. A similar phenomenon was also observed in prostate cancer cells. These results suggest that the duration of JNK activation may be the determining factor for the outcome of signaling. **The aims of this study are (i) to examine the role of the JNK pathway in proliferation apoptosis in prostate cancer cells, (ii) to study the mechanism of JNK-mediated apoptosis, (iii) to identified down-stream effectors involved in JNK-mediated apoptosis.**

BODY

The first two sections are the summaries of the studies that were initiated before July 1, 1999, and were completed during the funding period. Although these studies were not described in the proposal, both studies are related to the proposed research, and have been published (1,2).

1. JNK mediates apoptotic signaling induced by N-(4-hydroxyphenyl)retinamide (4-HPR) in prostate cancer cells — *These results were published in attached manuscript #1 (1)*

Retinoic acid and its synthetic analogs have diverse effects on development, morphogenesis, and homeostasis, and have been tested in the prevention and treatment of cancers (3,4). Natural retinoids show limited effects on many cancer cells; however, several synthetic retinoids exhibit potent biological activities. For example, N-(4-hydoxylphenyl) retinamide (4-HPR) shows promise in the treatment and prevention of several cancers (5,6). 4-HPR suppresses cell proliferation in cancer cells as does retinoic acid. However, 4-HPR induces apoptosis, while retinoic acid usually does not (7-10). The ability to arrest growth and to induce apoptosis gives 4-HPR great potential as an effective anticancer agent. To date, the mechanism by which 4-HPR induces apoptosis is poorly understood.

We examined the effects of 4-HPR on two prostate carcinoma cell lines, LNCaP (an androgen-sensitive, $p53^{+/+}$ cell line) and PC-3 (an androgen-insensitive, $p53^{-/-}$ cell line). 4-HPR caused sustained c-Jun N-terminal kinase (JNK) activation and apoptosis in LNCaP cells but not in PC-3 cells at the dosages tested. Activation of JNK by 4-HPR was independent of caspases, since a pan-caspase inhibitor failed to suppress JNK activation. Ultraviolet C and γ radiation induced JNK activation in both LNCaP and PC-3 cells, suggesting that the failure of PC-3 cells to respond to 4-HPR was due to defects upstream of the JNK pathway. Furthermore, γ radiation-induced JNK activation was suppressed by an antioxidant, but 4-HPR-induced JNK activation was not, indicating that these two stimuli induced JNK activation through different mechanisms. Forced expression of JNK1, but not a JNK1 mutant, caused apoptosis in both LNCaP and PC-3 cells, suggesting that p53 is not required for JNK-mediated apoptosis. 4-HPR-induced apoptosis in LNCaP cells was suppressed by curcumin, which inhibits JNK activation. Expression of dominant-negative mutants in the JNK pathway also inhibited 4-HPR-induced apoptosis in 293 cells. These results suggest that the JNK pathway mediates 4-HPR-induced apoptotic signaling.

The ability of an antioxidant (NAC) to block γ radiation-induced JNK activation, but not 4-HPR-induced JNK activation, indicates that these two agents induce JNK through distinct mechanisms. The activation of the JNK pathway by radiation but not by 4-HPR in PC-3 cells shows that genetic alterations in tumor cells may affect one but not other signaling pathways involved in the induction of JNK and apoptosis. Induction of apoptosis in PC-3 cells by forced expression of JNK1 suggests that we may be able to bypass the genetic defects in tumor cells that prevent apoptosis induction by activating JNK directly. The further examination on JNK-mediated apoptotic signaling will be important in the design of more effective cancer therapeutic agents.

2. Caspase-mediated cleavage of JNK-activating kinase HPK1 — *this study was published in attached manuscript #2 (2)*

Caspases, aspartate-directed cysteine proteases, are required for apoptosis. The blockage of caspase activation by peptide inhibitors or by viral proteins, such as the pox virus protein CrmA or baculovirus p35, suppresses apoptosis progression (reviewed in (11). Cleavage by caspases may enhance the biochemical activity of their substrates, e.g., caspases themselves (11) and protein kinase C δ (12). Cleavage by caspases can also diminish normal functions of the substrates, such as poly-(ADP-ribose) polymerase (PARP) (13), DNA-dependent protein kinase (14), MDM2 (15), $p21^{Cip1/Waf1}$, and $p27^{Kip1}$ (16). In addition, cleavage of nuclear lamin

(17), gelsolin (18), and focal adhesion kinase (FAK) (19) by caspases is involved in the morphological changes found in apoptotic cells.

Activation of c-Jun N-terminal kinase (JNK) by Fas ligation is caspase-dependent (20), suggesting that caspases may regulate activators of the JNK pathway. We found that an upstream activator of JNK, hematopoietic progenitor kinase 1 (HPK1), was cleaved during apoptosis. Cleavage of HPK1 was blocked by peptide inhibitors for caspases. HPK1 was efficiently processed by recombinant caspase 3 *in vitro*. A conserved caspase recognition site, DDVD (amino acid 382-385), was found in the HPK1 protein sequence. By testing HPK1 proteins with *in vivo* and *in vitro* cleavage assays, we showed that aspartic acid residue 385 is the target for caspases. HPK1 cleavage separated the amino (N)-terminal kinase domain from the carboxyl (C)-terminal regulatory domain, and enhanced HPK1 kinase activity. Unlike the full-length HPK1, the N-terminal cleaved product failed to bind adaptor molecules Grb2 (growth factor receptor-bound protein 2) and Crk (CT10 regulator of kinase). The C-terminal fragment, although having three proline-rich domains, bound to Grb2 and Crk less efficiently than the full-length HPK1 protein. Taken together, the cleavage of HPK1 by caspase profoundly changed its biochemical properties.

Recently, several JNK upstream activators including MEKK1, PAK2/hPAK65, and Mst1 were shown to be substrates of caspases (21-25). The similarities among these reports are that the cleavage separates the kinase domain from the regulatory domain, and enhances the kinase activity (21-25). The cleaved kinases have either the same or enhanced ability to activate JNK. In addition to HPK1, we found that other two HPK1-like, JNK activating kinases, GCK-like kinase (GLK) and HPK1/GCK homologous kinase (HGK) (26,27), were also cleaved by caspase activity during Fas-mediated apoptosis. Taken together, our and others' data suggest that the cleavage of JNK upstream regulators is a conserved mechanism, which may mediate JNK activation and other downstream effects. It has been shown that JNK activation is required for caspase activation by certain stimuli (28,29). It is possible that apoptotic signaling is a circuit that death stimuli can enter at either the JNK pathway or the caspase cascade, and the signaling circuit may amplify the death signal through the reciprocal interaction between these two signaling modules.

3. Induction of JNK by Mitogenic and Apoptotic Stimuli in Prostate Cancer Cells — *these experiments are related to specific aim #1 in the Statement of Work*

We found that the induction of JNK in response to mitogenic and apoptotic signals in prostate cancer LNCaP cells have different activation patterns, transient versus persistent, respectively. We treated LNCAP cells with either PMA or epidermal growth factor (EGF). Both PMA and EGF induce an immediate and transient JNK activation in LNCaP cells. The kinase activity increased in 15 min and decreased to basal levels in 90 min. Androgen treatment did not induce significant JNK activation in LNCaP cells. LNCAP were also treated with various apoptotic agents, and examined for endogenous JNK activation. Among the agents tested, γ radiation, UV-C, 4-HPR (see attached manuscript #1), and 5-FU showed strong JNK inducing ability. All of the apoptotic agents induced persistent JNK activation, which followed by apoptotic cell death as determined by morphological change and nuclear staining of the cells. These data reveal that mitogenic agents induced transient JNK activation in LNCaP cells, while apoptotic agents induced persistent JNK activation. These results are consistent with our previous observation in T cells, and further support our hypothesis that duration of JNK activation may determine cell fate.

Curcumin (diferuloylmethane), a dietary pigment from *Curcuma longa*, gives the golden-yellow color and unique flavor to curry. Previously, we found that curcumin may affect the JNK pathway by interfering with

the signaling molecule(s) at the same level or proximally upstream of the MAPKKK level (30). Curcumin treatment suppresses the proliferation of LNCaP cells in response to serum. Curcumin also suppress apoptosis induced by 4-HPR in LNCaP cells (1). These data suggest that the JNK pathway is required for both proliferation and apoptosis in prostate cancer cells.

4. Post-radiation treatment of curcumin, an inhibitor for JNK activation, fails to terminate JNK activation — *this work is related to specific aim #1 in the Statement of Work*

Since curcumin is an effective inhibitor for JNK activation by suppressing upstream kinases (30), we tested whether curcumin can be used to terminate JNK activation induced by apoptotic signals. If so, the association of duration of JNK induction and apoptosis can be determined. LNCAP cells were treated with UV-C. Twenty min post-irradiation, cells were incubated with or without curcumin. Although pre-incubation of curcumin inhibited JNK activation by all of the agonists tested (30). **Post-radiation treatment of curcumin failed to suppress the intensity or the duration of JNK activation.** This result suggests that once JNK has been activated, the activities of upstream kinase were not required for the sustained JNK activation, because of suppressing the upstream kinase by curcumin is not sufficient to turn off the JNK activation.

From this study, we conclude that curcumin can not be used to modulate the duration of JNK activation as proposed in the research proposal (Aim 1). However, a JNK specific inhibitor was recently reported (31). This inhibitor may be used to control the duration of JNK activation.

5. The role of Fas-Fas ligand in JNK-mediated apoptosis in prostate cancer cells — *this work is related to specific aim #2 in the Statement of Work.*

Previously, we have shown Fas expression induced by apoptotic stimuli is a p53-dependent phenomenon, but can be dissociated from JNK activation (32). Other investigators have reported that JNK activation leads to the expression of Fas ligand. Using various methods, we did not detect expression of Fas ligand in LNCaP prostate cancer, even in the presence of strong JNK activation. To further examine the relationship between Fas/Fas ligand and JNK-mediated apoptosis, we established radiation-resistant LNCaP cells by selecting LNCaP cells that survived lethal doses of γ radiation treatment. These radiation-resistant LNCaP cells did not respond to γ radiation with JNK activation and apoptotic cell death, which is consistent with the finding that JNK is required for γ radiation-induced apoptosis. However, these radiation-resistant cells were still sensitive to apoptosis induced by Fas ligation. Currently, the collective data indicate that the Fas-Fas ligand pathway is not required for radiation-induced and/or JNK-mediated apoptosis (32).

We are establishing LNCaP cells that are resistant to Fas-mediated apoptosis. We will test whether those cells are sensitive to JNK-mediated apoptosis. The radiation-resistant LNCaP cells will be used to identified the pathway(s) that are involved in JNK activation by γ radiation.

KEY RESEARCH ACCOMPLISHMENTS

- Showing that JNK is involved in and required for N-(4-hydroxyphenyl)retinamide-induced apoptosis in LNCaP cells (1)
- Showing that p53 tumor suppressor gene is not required for JNK-mediated apoptosis (1)
- Identification of hematopoietic progenitor kinase 1 (HPK1), a JNK-activating kinase, as a substrate for caspase 3 (2)
- Establishment of γ radiation-resistant LNCaP cells.

REPORTABLE OUTCOMES

Meeting Abstracts

1. Spore in Prostate Meeting, Apr. 14, 2000, Baylor College of Medicine, Houston; presentation entitled "The c-Jun N-terminal kinase pathway and apoptotic signaling in prostate cancer"

2. Keystone Symposium entitled "Advances in Human Breast and Prostate Cancer" Mar. 19-24, 2000, Lake Tahoe, Nevada; poster abstract (#203) entitled " c-Jun N-terminal kinase-mediated apoptotic signaling in prostate cancer"

Publications

1. Y.-R. Chen, C. F. Meyer, B. Ahmed, Z. Yao, and T.-H. Tan (1999) Caspase-mediated cleavage and functional changes of hematopoietic progenitor kinase 1 (HPK1). *Oncogene* **18**, 7370-7377

2. Y.-R. Chen, G. Zhou, and T.-H. Tan (1999) c-Jun N-terminal kinase mediates apoptotic signaling induced by N-(4-hydroxyphenyl)retinamide (4-HPR). *Mol. Pharmacol.*, **56**, 1271-1279

3. Y.-R. Chen and T.-H. Tan (2000) The c- Jun N-terminal kinase pathway and apoptotic signaling (Review). *Int. J. Oncol*, **16**, 651-662

Awards

1. 5/00 — Travel award (Advance in human breast and prostate cancer, Keystone symposium; NIH Grant#1 R13 CA85280-01)

CONCLUSION

Apoptosis plays a major role during development and homeostasis. Normal tissues are maintained by the balance between cell proliferation and apoptosis. Defects in apoptosis may result in cancer formation. Defects in apoptosis are among the leading causes of resistance to radiation and chemotherapy in cancer cells. Understanding the biochemical mechanisms of apoptosis is, therefore, very important for revealing cancer biology and for improving cancer therapies.

The c-Jun N-terminal kinase (JNK) pathway participates in cellular responses to both environmental stresses and mitogenic signals. We found that JNK is involved in cellular signaling during apoptosis induced by various agents including γ-radiation, UV-radiation, anti-carcinogenic isothiocyanates, and retinoid analog N-(4-hydroxyphenyl) retinamide (4-HPR) in various cancer cells (1,20,33). Interfering with the JNK pathway suppressed the induction of apoptosis. JNK activation by apoptotic stimuli can be caspase-dependent or independent. Different apoptotic stimuli induce JNK activation through distinct mechanisms. We found that oxidative stress is a major cause of JNK activation in apoptosis induced by several agents. The involvement of JNK in both mitogenic and apoptotic signaling implies that a subtle regulatory mechanism must exist for the signaling decision. Our studies reveal that the duration of JNK induction may determine cell fate. The mechanisms that regulate the duration of JNK activation are currently studied.

In conclusion, our study reveals the importance of the JNK pathway in apoptotic signaling (reviewed in attached review article 34). These results provide important information for the studies of oncogenesis and the mechanisms of radiation and drug resistance in cancer cells.

REFERENCES

1. Chen, Y.-R., Zhou, G., and T.-H., T. (1999) c-Jun N-terminal Kinase mediates apoptotic signaling induced by N-(4-hydroxyphenyl)retinamide. *Mol. Pharmacol.* **56,** 1271-1279
2. Chen, Y.-R., Meyer, C. F., Ahmed, B., Yao, Z., and Tan, T.-H. (1999) Caspase-mediated cleavage and functional changes of hematopoietic progenitor kinase 1 (HPK1). *Oncogene* **18,** 7370-7377
3. Lotan, R. (1996) Retinoids in cancer chemotherapy. *FASEB J.* **10,** 1031-1039
4. Means, A. L., and Gudas, L. J. (1995) The roles of retinoids in vertebrate development. *Ann. Rev. Biolchem.* **64,** 201-233
5. Kelloff, G. J., Crowell, J. A., Bonne, C. W., Steele, V. E., Lubet, R. A., Greenwald, P., Alberts, D. S., Covey, J. M., Doody, L. A., and Knapp, G. G. (1994) Clinical development plan: N-(4-hydroxylphenyl) retinamide. *J. Cell. Biochem., Supplement* **20,** 176-196
6. Pienta, K. J., Nguyen, N. M., and Lehr, J. E. (1993) Treatment of prostate cancer in the rat with the synthetic retinoid fenretinide. *Cancer Res.* **53,** 224-226
7. Delia, D., Aiello, A., Lombardi, L., Pelicci, P. G., Grignani, F., Grignani, F., Formelli, F., Menard, S., Costa, A., and Veronesi, U. (1993) N-(4-hydroxyphenyl) retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid. *Cancer Res.* **53,** 6036-6041
8. Fanjul, A. N., Delia, D., Pierotti, M. A., Rideout, D., Qiu, J., and Rfahl, M. (1996) 4-hydroxyphenyl retinamide is a highly selective activator of retinoid receptors. *J. Biol. Chem.* **271,** 22441-22446
9. Oridate, N., Lotan, D., Mitchell, M. F., Hong, W. K., and Lotan, R. (1995) Inhibition of proliferation and induction of apoptosis in cervical carcinoma cells by retinoids for chemotherapy. *Journal of Cellular Biochemistry, Supplement* **23,** 80-86
10. Ponzoni, M., Bocca, P., Chiesa, V., Decensi, A., Pistoia, V., Raffaghello, L., Rozzo, C., and Montaldo, P. G. (1995) Differential effects of N-(4-hydroxyphenyl) retinamide and retinoic acid on neuroblastoma cells: apoptosis versus differentiation. *Cancer Res.* **55,** 853-861
11. Salvesen, G., and Dixit, V. M. (1997) Caspases: intracellular signaling by proteolysis. *Cell* **91,** 443-446
12. Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbabda, S., Robertson, M., Ghayur, T., Wong, W. W., Kamen, R., Weichselbaum, R., and Kufe, D. (1995) Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. *EMBO J* **14,** 6148-6156
13. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371,** 346-347
14. Song, O., Lees-Miller, S. P., Kumar, S., Zhang, N., Chan, D. W., Smith, G. C. M., Jackson, S. P., Alnemri, E. S., Litwack, G., Khanna, K. K., and Lavin, M. F. (1996) DNA-dependent protein kinase catalytic subunit; a target for an ICE-like protease in apoptosis. *EMBO J.* **15,** 3238-3246
15. Erhardt, P., Tomaselli, K. J., and Cooper, G. M. (1997) Identification of the MDM2 oncoprotein as a substrate for COO32-like apoptotic proteases. *J. Biol. Chem.* **272,** 15049-15052
16. Levkau, B., Koyama, H., Raines, E. W., Clurman, B. E., Herren, B., Orth, K., and Roberts, J. M. (1998) Cleavage of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ mediates apoptosis in endothelial cells through activation of Cdk2:role of a caspase cascade. *Mol. Cell* **1,** 553-563
17. Lazebnik, Y. A., Takahashi, R. D., Moir, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H., and Earnshaw, W. C. (1995) Studies of the lamin proteinase reveal mutiple parallel biochemical pathways during apoptotic execution. *Proc. Natl. Acad. Sci. USA* **92,** 9042-9046
18. Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T. J., Kirschner, M. W.,

Koths, K., Kwiatkowski, D. J., and Williams, L. T. (1997) Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278,** 294-298

19. Wen, L.-P., Fahrni, J. A., Troie, S., Guan, J.-L., Orth, K., and Rosen, G. D. (1997) Cleavage of focal adhesion kinase by caspases during apoptosis. *J. Biol. Chem.* **272,** 26056-26061
20. Chen, Y.-R., Wang, X., Templeton, D., Davis, R. J., and Tan, T.-H. (1996) The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and γ radiation: Duration of JNK activation may determine cell death and proliferation. *J. Biol. Chem.* **271,** 31929-31936
21. Cardone, M., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1997) The regulation of anoikis: MEKK1 activation requires cleavage by caspases. *Cell* **90,** 315-323
22. Widmann, C., Gerwins, P., Johson, N. L., Jarpe, M. B., and Johnson, G. L. (1998) MEK kinase 1, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. *Mol. Cell. Biol.* **18,** 2416-2429
23. Deak, J. C., Cross, J. V., Lewis, M., Qian, Y., Parrott, L. A., Distelhorst, C. W., and Templeton, D. J. (1998) Fas-mediated proteolytic activation and intracellular redistribution of the stress-signaling kinase MEKK1. *Proc. Natl. Acad. Sci. USA* **95,** 5595-5600
24. Rudel, T., and Bokoch, G. M. (1997) Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation PAK2. *Science* **276,** 1571-1574
25. Graves, J. D., Gotoh, Y., Draves, K., Ambrose, D., Han, D. K. M., Wright, M., Chernoff, J., Clark, E. A., and Krebs, E. G. (1998) Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like Mst1. *EMBO J.* **17,** 2224-2234
26. Diener, K., Wang, X. S., Chen, C., Meyer, C. F., Bray, J., Zukowski, M., Tan, T.-H., and Yao, Z. (1997) Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase. *Proc. Natl. Acad. Sci. USA* **94,** 9687-9692
27. Yao, Z., Zhou, G., Wang, X. S., Brown, A., Diener, K., Gan, H., and Tan, T.-H. (1999) A novel mammalian STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. *J. Biol. Chem.* **274,** 2118-2125
28. Seimiya, H., Mashima, T., Toha, M., and Tsuruo, T. (1997) c-Jun N-terminal kinase-mediated activation of the interleukin-1β converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis. *J. Biol. Chem.* **272,** 4631-4636
29. Shiah, S.-G., Chuang, S.-E., Chau, Y.-P., Shen, S.-C., and Kuo, M.-L. (1998) Activation of c-Jun NH_2-terminal kinase and subsequent CPP32/YAMA during topoisomerase inhibitor β-lapachone-induced apoptosis through an oxidative-dependent pathway. *Cancer Res.* **59,** 391-398
30. Chen, Y.-R., and Tan, T.-H. (1998) Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene* **17,** 173-178
31. Xia, Y., Makris, C., Su, B., Li, E., Yang, J., Nemerow, G., and Karin, M. (2000) MEK kinase 1 is critically required for c-Jun N-terminal kinase activation by proinflammatory stimuli and growth factor-induced cell migration. *Proc. Natl. Acad. Sci. USA* **97,** 5243-5248
32. Chen, Y.-R., and Tan, T.-H. (1999) Lack of correlation in JNK activation and p53-dependent Fas expression induced by apoptotic stimuli. *Biochem. Biophys. Res. Comm.* **256,** 595-599
33. Chen, Y.-R., Wang, W., Kong, A.-N. T., and Tan, T.-H. (1998) Molecular mechanisms of c-Jun N-terminal kinase (JNK)-mediates apoptosis induced by anticarcinogenic isothiocyanates. *J. Biol. Chem.* **273,** 1769-1775

34. Chen, Y.-R., and Tan, T.-H. (2000) The c-Jun N-terminal kinase pathway and apoptotic signaling. *Int. J. Oncol.* **16,** 651-662

APPENDICES

Abstarcts

1. "The c-Jun N-terminal kinase pathway and apoptotic signaling in prostate cancer", presented in Spore in Prostate Meeting, Mar. 23, 2000, Baylor College of Medicine, Houston, Texas

2. " c-Jun N-terminal kinase-mediated apoptotic signaling in prostate cancer", presented in Keystone Symposium entitled "Advances in Human Breast and Prostate Cancer" Mar. 19-24, 2000, Lake Tahoe, Nevada

Reprints of Manuscripts

1. Y.-R. Chen, C. F. Meyer, B. Ahmed, Z. Yao, and T.-H. Tan (1999) *Oncogene* **18**, 7370-7377

2. Y.-R. Chen, G. Zhou, and T.-H. Tan (1999) *Mol. Pharmacol.*, **56**, 1271-1279

3. Y.-R. Chen and T.-H. Tan (2000) *Int. J. Oncol*, **16**, 651-662

SPORE IN PROSTATE - BAYLOR COLLEGE OF MEDICINE

ABSTRACT FORM

2000 INTERNAL ADVISORS ANNUAL SCIENTIFIC MEETING

DEADLINE FOR SUBMITTING PAPERS IS MARCH 23, 2000

Mail the original and 6 copies in a flat envelope to:
Linda Higgins, SPORE Coordinator, Baylor College of Medicine, F- 403
See attached sheet for complete instructions before typing abstract ***PLEASE INDICATE PRESENTOR***

The C-JUN N-TERMINAL KINASE PATHWAY AND APOPTOTIC SIGNALING IN PROSTATE CANCER

Yi-Rong Chen, Guisheng Zhou, and Tse-Hua Tan, Department of Microbiology and Immunology, Baylor College of Medicine, Houston, TX 77030

Apoptosis plays a major role in maintaining homeostasis in prostate tissues. Defects in apoptosis may result in prostate hyperplasia or prostate cancer. Defects in apoptosis are leading causes of resistance to radiation and chemotherapy in prostate cancer. Understanding the biochemical mechanisms of apoptosis is, therefore, very important for revealing prostate cancer biology and for improving cancer therapies. c-Jun N-terminal Kinase (JNK) participates in cellular responses to both environmental stresses and mitogenic signals. Previously, we found that JNK is involved in apoptotic signaling induced by various agents including γ-radiation, UV-radiation, and chemopreventive and therapeutic agents for cancer.

Transforming growth factor β (TGF-β), a factor that is involved in the development of prostate cancer, induces JNK activation via TGF-β activated kinase 1 (TAK1). We identified hematopoietic progenitor kinase 1 (HPK1) as an upstream kinase for TAK1 in TGF-β-induced JNK activation. HPK1 is activated by TGF-β, and JNK activation by TGF-β is suppressed by a dominant-negative HPK1 mutant. TGF-β enhances the association between HPK1 and TAK1, and HPK1-induced JNK activation is blocked by a dominant-negative TAK1 mutant. The effect of TGF-β on JNK activation was examined in three prostate cancer cell lines. TGF-β failed to induce JNK activation in LNCaP cells. This result is consistent with the reports showing the genetic defects in TGF-β receptors in LNCaP cells. PC-3 and DU145 responded to TGF-β with different patterns of JNK activation, transient and persistent, respectively. The molecular mechanism underlies the different JNK activation patterns was currently under investigation.

N-(4-hydroxyphenyl) retinamide (4-HPR), a retinoic acid analog, caused sustained c-Jun N-terminal kinase (JNK) activation and apoptosis in LNCaP cells but not in PC-3 cells. Activation of JNK by 4-HPR was independent of caspases, since a pan-caspase inhibitor failed to suppress JNK activation. Ultraviolet C and γ radiation induced JNK activation in both LNCaP and PC-3 cells, suggesting that the failure of PC-3 cells to respond to 4-HPR was due to defects upstream of the JNK pathway. Furthermore, γ radiation-induced JNK activation was suppressed by an antioxidant, but 4-HPR-induced JNK activation was not, indicating that these two stimuli induced JNK activation through different mechanisms. By interfering with the JNK signaling, we demonstrated that JNK activation is important for 4-HPR-induced apoptotic signaling.

We identified several actin-binding and SH3 domain-containing proteins that can interact with HPK1 through their SH3 domains. The association between these proteins and HPK1 may participate in JNK signaling. Interestingly, these actin-binding proteins were cleaved by caspases in apoptotic prostate cancer cells. The cleavage of these proteins may be involved in the reorganization of cytoskeleton and morphological changes in apoptotic cells.

TO WHOM SHOULD CORRESPONDENCE BE DIRECTED? (Please type) **Presenter's Name:** Yi-Rong Chen

Name: Tse-Hua Tan Degree(s): Ph.D. Role on Project: Principal Investigator

Phone Number: 798-4665 Mail Station: M929 Department: Immunology

c-Jun N-terminal Kinase-mediated Apoptotic signaling in Prostate Cancer

Yi-Rong Chen, Guisheng Zhou, and Tse-Hua Tan, Department of Microbiology and Immunology, Baylor College of Medicine, Houston, TX 77030

Apoptosis plays a major role during development and homeostasis. Normal prostate tissue is maintained by the balance between cell proliferation and apoptosis. Defects in apoptosis may result in prostate hyperplasia or prostate cancer. Defects in apoptosis are among the leading causes of resistance to radiation and chemotherapy in prostate cancer cells. Understanding the biochemical mechanisms of apoptosis is, therefore, very important for revealing prostate cancer biology and for improving cancer therapies.

c-Jun N-terminal Kinase (JNK) participates in cellular responses to both environmental stresses and mitogenic signals. We found that JNK is involved in cellular signaling during apoptosis induced by various agents including γ-radiation, UV-radiation, and retinoid analog N-(4-hydroxyphenyl)retinamide (4-HPR) in prostate cancer cells. Iinterfering with the JNK pathway suppressed the induction of apoptosis. Activation of JNK by these stimuli are caspase-independent. Different apoptotic stimuli induce JNK activation through distinct mechanisms.

The involvement of JNK in both mitogenic and apoptotic signaling implies that a subtle regulatory mechanism must exist for the signaling decision. Our previous studies reveal that the duration of JNK induction may determine cell fate. The mechanisms that regulate the duration of JNK activation are currently studied. We have identified and cloned several JNK-activating, STE20-related kinases including HPK1, HGK and GLK. The potential roles of these kinases mitogenic and/or apoptotic signaling in prostate cancer cells will be examined.

0026-895X/99/061271-09$3.00/0

MOLECULAR PHARMACOLOGY, 56:1271–1279 (1999).

c-Jun N-Terminal Kinase Mediates Apoptotic Signaling Induced by *N*-(4-Hydroxyphenyl)retinamide

YI-RONG CHEN, GUISHENG ZHOU, and TSE-HUA TAN

Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas

Received March 22, 1999; accepted August 25, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

N-(4-Hydroxyphenyl)retinamide (4-HPR), a retinoic acid analog, induces apoptosis in several cell types. The mechanism by which 4-HPR initiates apoptosis remains poorly understood. We examined the effects of 4-HPR on two prostate carcinoma cell lines, LNCaP (an androgen-sensitive, $p53^{+/+}$ cell line) and PC-3 (an androgen-insensitive, $p53^{-/-}$ cell line). 4-HPR caused sustained c-Jun N-terminal kinase (JNK) activation and apoptosis in LNCaP cells but not in PC-3 cells at the dosages tested. Activation of JNK by 4-HPR was independent of caspases because a pan-caspase inhibitor failed to suppress JNK activation. Ultraviolet-C and γ-radiation induced JNK activation in both LNCaP and PC-3 cells, suggesting that the failure of PC-3 cells to respond to 4-HPR was due to defects upstream of the JNK pathway. Furthermore, γ-radiation-induced JNK activation was suppressed by an antioxidant, but 4-HPR-induced JNK activation was not, indicating that these two stimuli induced JNK activation through different mechanisms. Forced expression of JNK1, but not a JNK1 mutant, caused apoptosis in both LNCaP and PC-3 cells, suggesting that p53 is not required for JNK-mediated apoptosis. 4-HPR-induced apoptosis in LNCaP cells was suppressed by curcumin, which inhibits JNK activation. Expression of dominant-negative mutants in the JNK pathway also inhibited 4-HPR-induced apoptosis in human embryonic kidney 293 cells. Collectively, these results suggest that the JNK pathway mediates 4-HPR-induced apoptotic signaling.

Retinoic acid and its synthetic analogs have diverse effects on development, morphogenesis, and homeostasis, and have been tested in the prevention and treatment of cancers (Means and Gudas, 1995; Lotan, 1996). Natural retinoids show limited effects on many cancer cells; however, several synthetic retinoids exhibit potent biological activities. For example, *N*-(4-hydoxylphenyl)retinamide (4-HPR) shows promise in the treatment and prevention of several cancers (Pienta et al., 1993; Kelloff et al., 1994). 4-HPR suppresses cell proliferation in cancer cells as does retinoic acid. However, 4-HPR induces apoptosis, whereas retinoic acid usually does not (Delia et al., 1993; Oridate et al., 1995; Ponzoni et al., 1995; Fanjul et al., 1996). The ability to arrest growth and to induce apoptosis gives 4-HPR great potential as an effective anticancer agent. To date, the mechanism by which 4-HPR induces apoptosis is poorly understood.

c-Jun N-terminal kinase (JNK; also named stress-activated protein kinase) is a member of the mitogen-activated protein kinase (MAPK) family, which also includes extracellular signal-regulated kinase and p38-MAPK. JNK was first identified by its ability to respond to environmental stresses, proinflammatory cytokines, and mitogens (for review, see Kyriakis and Avruch, 1996; Ip and Davis, 1998). The JNK pathway was later found to be important in apoptosis signaling (for review, see Ip and Davis, 1998). Interference with the JNK pathway suppresses the induction of apoptosis by various agents (Xia et al., 1995; Chen et al., 1996b, 1998; Verheij et al., 1996; Zanke et al., 1996). JNK phosphorylates the Ser63/Ser73 residues in the N-terminal *trans*-activating domain of c-Jun, strongly augmenting its transcriptional activity (Whitmarsh and Davis, 1996). In addition, the JNK pathway activates activating transcription factor-2 (Gupta et al., 1995), Elk-1 (Whitmarsh et al., 1995), and Sap-1a transcription factors (Janknecht and Hunter, 1997), and interacts with the nuclear factor-κB pathway (Meyer et al., 1996; Lee et al., 1997). The mechanisms by which the JNK pathway participates in these diverse cellular functions are unclear. Our previous data suggest that the duration of JNK activation determines cell fate (Chen et al., 1996a,b). In this study, we examined the apoptotic effect of 4-HPR on two prostate carcinoma cell lines, LNCaP and PC-3. We found that 4-HPR induced sustained JNK activation and apoptosis in LNCaP

This work was supported by National Institutes of Health Grants R01-AI38649 and R01-AI42532 (to T.-H.T.). Y.-R.C. was supported by Department of Defense Predoctoral Fellowship DAMD17-97-1-7078 in the Breast Cancer Research Program and is a recipient of Department of Defense Postdoctoral Fellowship DAMA17-99-1-9507) in the Prostate Cancer Research Program. T.-H.T. is a Scholar of the Leukemia Society of America.

ABBREVIATIONS: 4-HPR, *N*-(4-hydroxyphenyl)retinamide; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; GST, glutathione *S*-transferase; z-VAD-FK, z-Val-Ala-Asp-fluoromethyl ketone; UV-C, ultraviolet C; X-gal, 5-bromo-4-chloro-3-indolyl-β-galactopyranoside; NAC, *N*-acetyl-L-cysteine; TGF, transforming growth factor; HEK293 cells, human embryonic kidney 293 cells.

but not in PC-3 cells. Interference with the JNK signaling suppressed apoptosis induced by 4-HPR. Our results suggest that the JNK pathway mediates apoptotic signaling induced by 4-HPR.

Materials and Methods

Cells and Antibodies. The culture of human embryonic kidney 293 cells (HEK293 cells) was described previously (Chen et al., 1998). The prostate carcinoma cell lines LNCaP and PC-3 were kindly provided by K.-M. Tchou-Wong (New York University Medical School, New York, NY), and were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and streptomycin/penicillin. The rabbit anti-JNK1 antibody Ab101 was described previously (Chen et al., 1996a). The rabbit anti-p38-MAPK antibody Ab221 was generated against the peptide containing the carboxy-terminal 18 amino acids (DEVISFVPPPLDQEEMES) of p38 kinase. Anti-caspase 3 (anti-p11; no. K-19), anti-Bcl-2 (no. 100), and anti-Bcl-X_L (no. S-18) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-HA (12CA5) antibody was purchased from Boehringer Mannhein Corp. (Indianapolis, IN). Anti-Flag (M2), anti-glutathione *S*-transferase (GST), and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

Reagents and Radiation Treatments. 4-HPR was purchased from Sigma Chemical Co. and prepared as concentrated stock solutions in ethanol. SB202190 and curcumin were obtained from Calbiochem Corp. (La Jolla, CA) and Sigma Chemical Co., respectively, and dissolved in dimethyl sulfoxide. *N*-Acetyl-L-cysteine was obtained from Sigma Chemical Co. The caspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone (z-VAD-FK) was obtained from Kamiya Biomed. Co. (Thousand Oaks, CA). Ultraviolet-C (UV-C) irradiation was performed with a UV Stratalinker 1800 from Stratagene, Inc. (La Jolla, CA). Gamma irradiation was performed with a Gammacell 1000 ^{137}Cs source (Atomic Energy of Canada Ltd., Commercial Products, Ottawa, Ontario, Canada).

Plasmids. The GST-Jun(1–79) plasmid was described previously (Chen et al., 1996b). The pHA-JNK1 plasmid was provided by Dr. J. R. Woodgett (Ontario Cancer Institute, Toronto, Canada) (Pombo et al., 1995; Yao et al., 1997). The pEBG-GST-SEK1(AL) was provided by L. I. Zon (Children's Hospital, Boston, MA; Pombo et al., 1995), pcDNA3-Flag-JNK1(APF) was provided by R. J. Davis (University of Massachusetts, Worcester, MA; Gupta et al., 1995).

Apoptosis Assays. For nuclear morphology staining, the harvested cells were fixed with 1% paraformaldehyde (in 1× PBS) for 10 min, washed once with 1× PBS, then stained with Hoechst 33258 (2.5 ng/ml in PBS). The nuclear morphology was examined with a fluorescence microscope, and cells with condensed or fragmented nuclei were identified as apoptotic cells. For flow cytometry analyses of DNA staining profile, 5×10^5 or 10^6 cells were collected and washed with PBS once, then fixed with 70% ethanol. Fixed cells were washed with PBS to remove residual ethanol, pelleted, and resuspended in PBS containing propidium iodide (10 μg/ml; Sigma Chemical Co.). The stained cells were analyzed by flow cytometry (model XL; Coulter Corp., Hialeah, FL). Forward light scatter characteristics were used to exclude the cell debris from the analysis. Apoptotic cells were determined by their hypochromic, subG1 staining profiles. DNA fragmentation assays were performed as described in Herrmann et al. (1994).

Cell Extract Preparation and Immunocomplex Kinase Assays. Whole cell extract was prepared by suspending 2×10^6 cells in 200 μl of lysis buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4]. The cell lysate was kept on ice for 20 min and vigorously mixed in a Vortex mixer every 5 min. The lysate was cleared by centrifugation at 15,000*g* for 3 min, and the supernatant was stored at −80°C. JNK assays were carried out as described in Chen et al. (1996b). For p38-MAPK assays, endogenous p38-MAPK was precipitated by incubation with an anti-p38 antibody (Ab221) and protein A-agarose beads (Bio-Rad Laboratories, Inc., Richmond, CA) in the lysis buffer at 4°C for 3 h. The precipitates were washed twice with the lysis buffer and twice with kinase buffer [25 mM HEPES (pH 7.6), 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mM Na_3VO_4], then mixed with 5 μg of myelin basic protein, 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP in 30 μl of kinase buffer. The kinase reaction was per-

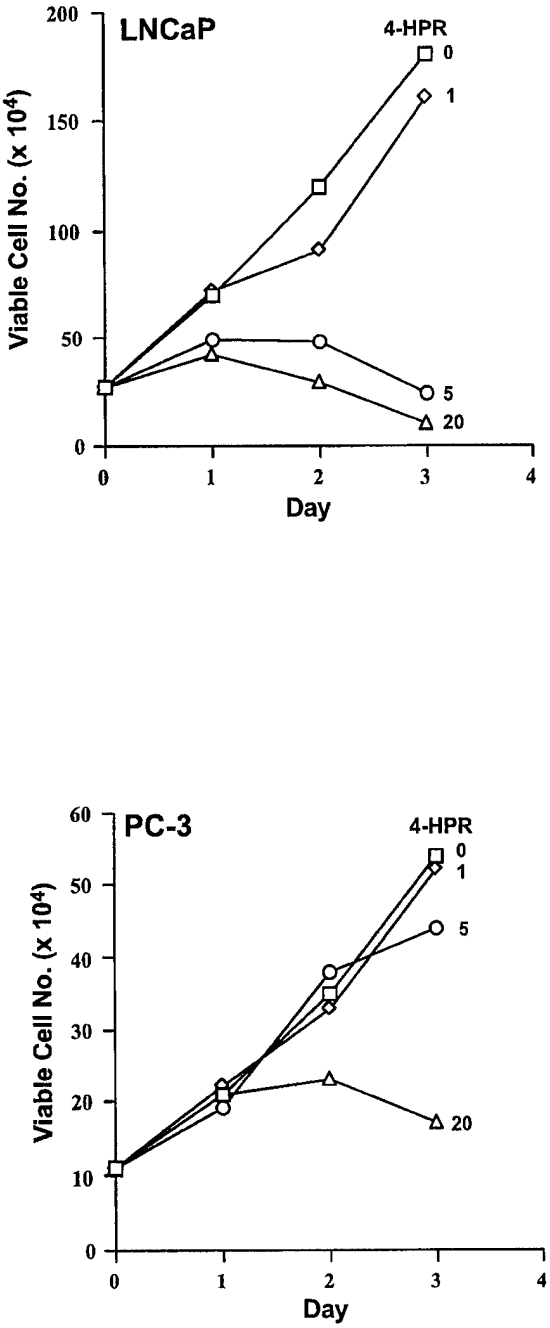
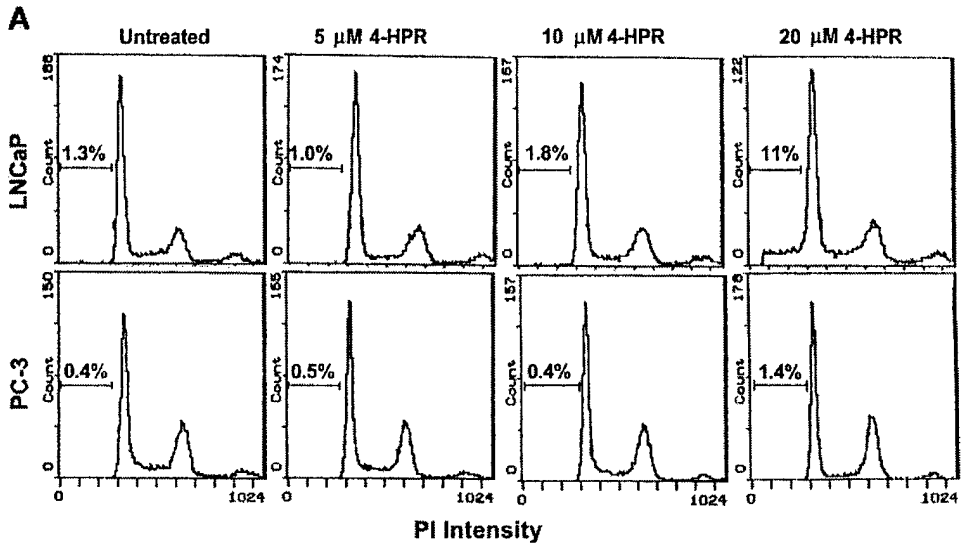
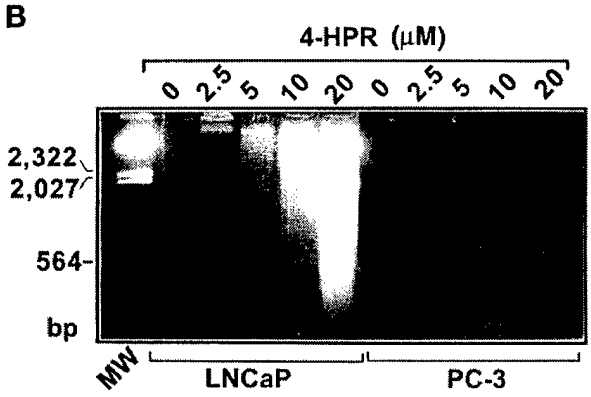


Fig. 1. 4-HPR decreases cell viability in LNCaP and PC-3 cells. LNCaP (A) and PC-3 cells (B) were plated in six-well plates 24 h before treatment. The cells were treated with 4-HPR at the indicated concentrations on day 0, and harvested on days 1, 2, and 3. Viable cells per well were determined by the trypan blue exclusion assay.

formed at 30°C for 30 min, then terminated by adding SDS-sample buffer. The reaction mixtures were boiled and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Transient Transfection-Protection/Apoptosis Assays. Transient transfection-protection assays were performed as described with modifications (Chen et al., 1996b); HEK293 cells were plated in a 35-mm-well plate (1.5×10^5 cells/well) the day before transfection. Cells were transfected with plasmids encoding β-galactosidase (1 μg) in combination with empty vector or the indicated plasmids encoding dominant-negative kinase mutants (2 μg for each). The transfections were performed by a calcium phosphate precipitation method (Specialty Media, Phillipsburg, NJ). Transfected cells were cultured in complete medium for 6 h after removing the transfection mixture, and then treated with 4-HPR (20 μM) or left untreated for 12 h. Cells were fixed in 1% paraformaldehyde for 10 min, washed twice with PBS, and stained with the staining solution [PBS (pH 7.4), 1 mM $MgCl_2$, 10 mM $K_4[Fe(CN)_6]$, 10 mM $K_3[Fe(CN)_6]$, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal)]. Transfected cells (blue color) with rounding up, shrinkage, or membrane-blebbing morphology were identified as apoptotic cells. Apoptosis induction was represented as percentage of apoptotic cells per 300 blue cells. For transient transfection-apoptosis assays, LNCaP or PC-3 cells were transfected with plasmids encoding β-galactosidase (3 μg) in combination with the indicated kinase plasmids as





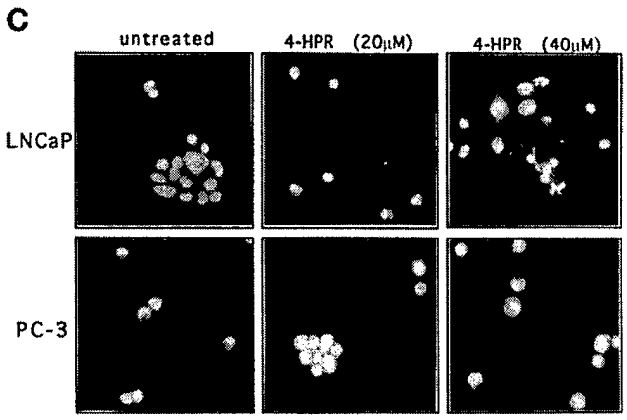


Fig. 2. 4-HPR induces apoptosis in LNCaP but not in PC-3 cells. LNCaP and PC-3 cells were treated with 4-HPR at the indicated concentrations. A, cells were harvested 36 h post-treatment, fixed, stained with propidium iodide (PI), and analyzed for DNA staining profiles as described in *Materials and Methods*. B, cells were harvested 36 h post-treatment and analyzed for DNA fragmentation as described in *Materials and Methods*. MW, λ-DNA/*Hin*dIII markers. C, LNCaP and PC-3 cells were harvested 24 and 48 h, respectively, after treatment. Harvested cells were fixed, stained, and examined for nuclear morphology.

described in figure legends. Cells were stained with X-gal 24 or 48 h after the transfections, and apoptosis induction was measured as described above. Two hundred transfected cells (blue color) were examined in every transfection.

Western Blot Assays. The cells were lysed as described above. The lysate was resolved by SDS-polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride membrane. The membrane was then incubated with a primary antibody (anti-caspase 3, 1:200 dilution; anti-Bcl-2, anti-Bcl-X_L, and anti-GST, 1:1000; anti-HA, 1 μg/ml; anti-Flag, 5 μg/ml), washed, and blotted with a secondary antibody conjugated with horseradish peroxidase (1:1000 dilution). The membrane was then developed in the enhanced chemiluminescence reagent from Amersham (Piscataway, NJ) and exposed to an X-ray film.

Results

4-HPR Induces Apoptosis in LNCaP Cells But Not in PC-3 Cells. Two prostate carcinoma cell lines, LNCaP and PC-3, were treated with various concentrations of 4-HPR and were examined by trypan blue exclusion assays at different time points after treatment. 4-HPR had a strong suppressive effect on LNCaP cell growth. At concentrations >5 μM, 4-HPR effectively decreased the growth of LNCaP cells in 1-day cultures (Fig. 1A), and this inhibitory effect was more evident at higher concentrations of 4-HPR or at later time points (Fig. 1A). In contrast, PC-3 cells were more resistant to 4-HPR treatment because low concentrations of 4-HPR (1 and 5 μM) had no apparent effect on cell viability (Fig. 1B). 4-HPR (20 μM) effectively suppressed PC-3 cell growth after a 2-day incubation (Fig. 1B).

4-HPR induces apoptosis in several cancer cell lines, including breast cancer cells (Fanjul et al., 1996), leukemia cells (Delia et al., 1993), cervical carcinoma cells (Oridate et al., 1995), and neuroblastoma cells (Ponzoni et al., 1995). Decreases of cell viability in LNCaP and PC-3 cells after 4-HPR treatment (Fig. 1) suggest that 4-HPR may induce apoptosis in these two prostate carcinoma cells. Various assays were performed to examine the ability of 4-HPR to induce apoptosis in LNCaP and PC-3 cells. With flow cytometry, we detected apoptosis induction, as defined by the appearance of cells with a sub-G1 staining pattern, in LNCaP cells treated with 20 μM 4-HPR (Fig. 2A). No significant apoptosis was detected in PC-3 treated with various doses of 4-HPR (Fig. 2A). DNA fragmentation also was observed in 4-HPR-treated LNCaP cells in a dose-dependent manner, but not in 4-HPR-treated PC-3 cells (Fig. 2B). In addition, 4-HPR (20 μM) caused nuclear condensation and fragmentation in LNCaP cells (Fig. 2C), but it failed to do so in PC-3 cells (Fig. 2C).

Caspase 3 is an important mediator in the apoptotic pathway, and the cleavage of caspase 3 is a hallmark of apoptosis in various cell types. By Western blot analyses, we also observed the processing of caspase 3 in 4-HPR-treated LNCaP cells but not in PC-3 cells (Fig. 3, A and B). Collectively, our results indicate that LNCaP cells were more sensitive to 4-HPR-induced apoptosis than were PC-3 cells. Although 4-HPR was capable of suppressing PC-3 cell growth (Fig. 1B), it did not cause apparent apoptosis in PC-3 cells at concentrations tested.

4-HPR Induces JNK Activation in LNCaP Cells But Not in PC-3 Cells. Although 4-HPR effectively induces apoptosis in various cell types, the biochemical mechanism is unclear. Recently, the JNK kinase pathway was shown to play an important role in apoptosis signaling (Xia et al., 1995; Chen et al., 1996b, 1998; Verheij et al., 1996; Zanke et al., 1996). We decided to examine whether the JNK pathway participates in 4-HPR-induced apoptosis. In LNCaP cells treated with 4-HPR (20 μM), JNK activity was detected after the 6-h time point. The kinase activity plateaued at 14 h and remained elevated up to 36 h after treatment (Fig. 4, A and B). 4-HPR failed to induce JNK in PC-3 cells (Fig. 4A), as would be expected from the absence of apoptosis (Figs. 2 and 3). We did not observe significant p38-MAPK activation in either LNCaP (Fig. 4C) or PC-3 cells (data not shown) treated with 4-HPR. These results show an association between JNK activation and apoptosis induction in these two prostate carcinoma cells.

Caspases are important effector molecules in apoptotic process. To establish the molecular order between the JNK pathway and caspases, we examined JNK induction by 4-HPR in the presence or absence of a pan-caspase inhibitor, z-VAD-FK, which suppresses the activation of multiple caspases. The caspase inhibitor failed to suppress 4-HPR-induced JNK activation, although it blocked the processing of caspase 3 (Fig. 4B). In addition, JNK activation preceded the cleavage of caspase 3 in 4-HPR-treated LNCaP cells (Figs. 3A and 4A). These data suggest that JNK activation by 4-HPR is independent of caspase activation and, therefore, should be an initiating signal rather than an end effect of 4-HPR-induced apoptosis.

Radiation and 4-HPR Induce JNK Activation through Different Pathways. The differential induction of JNK and apoptosis by 4-HPR in LNCaP and PC-3 cells sug-

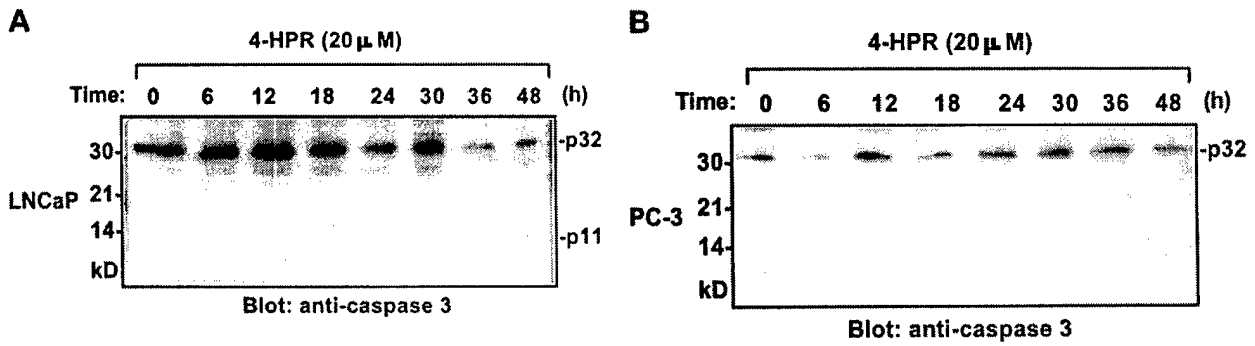


Fig. 3. 4-HPR induces cleavage of caspase 3 in LNCaP but not in PC-3 cells. LNCaP (A) and PC-3 cells (B) were treated with 4-HPR (20 μM) and harvested at the indicated time points; endogenous caspase 3 was examined by Western blotting with a specific antibody.

gests that the failure of 4-HPR to induce JNK activation and apoptosis in PC-3 cells may result from defects in apoptotic signaling. To determine whether the JNK pathway is functional in PC-3 cells, we used γ-radiation and UV-C, strong JNK and apoptosis inducers, to activate the JNK pathway. Both γ-radiation and UV-C induced JNK activation in PC-3 cells, although the activation was slower than that in LNCaP cells (Fig. 5, A and B). The radiation-induced apoptosis was detected by examination of morphological changes 24 to 48 h after irradiation (data not shown). This result shows that the JNK pathway in PC-3 cells is responsive to the radiation treatments. Therefore, the JNK pathway is functional in PC-3 cells, and the failure of these cells to respond to 4-HPR may be due to other defects in cellular signaling upstream of JNK.

We and others have shown that oxidative stress induces JNK activation in apoptosis induced by γ-radiation, anticancer agents, and growth factor withdrawal (Park et al., 1996; Chen et al., 1998). Therefore, we examined whether 4-HPR-induced JNK activation is mediated through oxidative stress. Cotreatment of LNCaP cells with 4-HPR and an antioxidant, *N*-acetyl-L-cysteine (NAC), did not block JNK activation by 4-HPR (Fig. 5C). In contrast, NAC completely suppressed γ-radiation-induced JNK activation. This result indicates that, unlike γ-radiation-induced JNK activation, the 4-HPR-induced JNK activation is not mediated through oxidative stress. Collectively, these data suggest that γ-radiation and 4-HPR use different pathways to induce JNK activation.

Expression of Bcl-2 Was Decreased in 4-HPR-Induced Apoptosis in LNCaP Cells. Expression of antiapoptotic molecules, such as Bcl-2 and Bcl-X_L, is associated with resistance to apoptotic stimuli (for review, see Adams and Cory, 1998). We examined the correlation between the expression of antiapoptotic molecules Bcl-2 and Bcl-X_L and the susceptibility to 4-HPR-induced apoptosis in LNCaP and PC-3 cells. These two cell lines expressed comparable levels of Bcl-X_L, however, Bcl-2 expression was slightly higher in

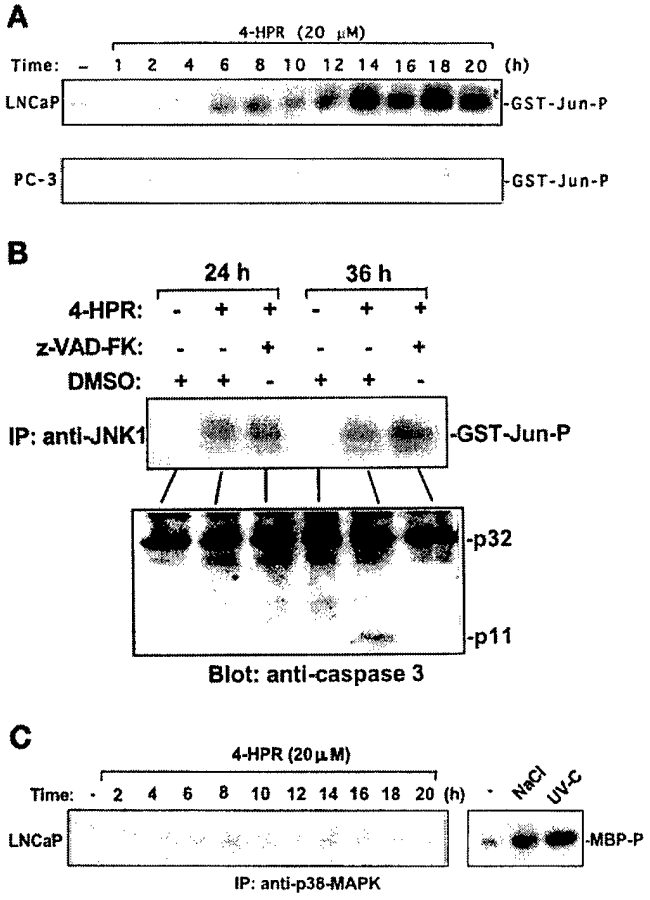


Fig. 4. 4-HPR induces persistent JNK activation in LNCaP but not in PC-3 cells. A, LNCaP and PC-3 cells were treated with 20 μM 4-HPR. The cells were collected at the indicated time points, and the endogenous JNK activity was determined by immunocomplex kinase assays. B, LNCaP cells treated with 20 μM 4-HPR in the presence or absence of the caspase inhibitor, z-VAD-FK, for 24 or 36 h. Endogenous JNK activity was examined by immunocomplex kinase assays, and endogenous caspase 3 was examined by Western blotting with a specific antibody. C, LNCaP cells were treated with 20 μM 4-HPR, osmotic shock (250 mM NaCl; 30 min), or UV-C (200 J/m^2). The cells were collected at the time points indicated and endogenous p38-MAPK activity was determined by immunocomplex kinase assays with myelin basic protein as a substrate. Activation of p38-MAPK by osmotic shock and UV-C served as controls for p38-MAPK assays.

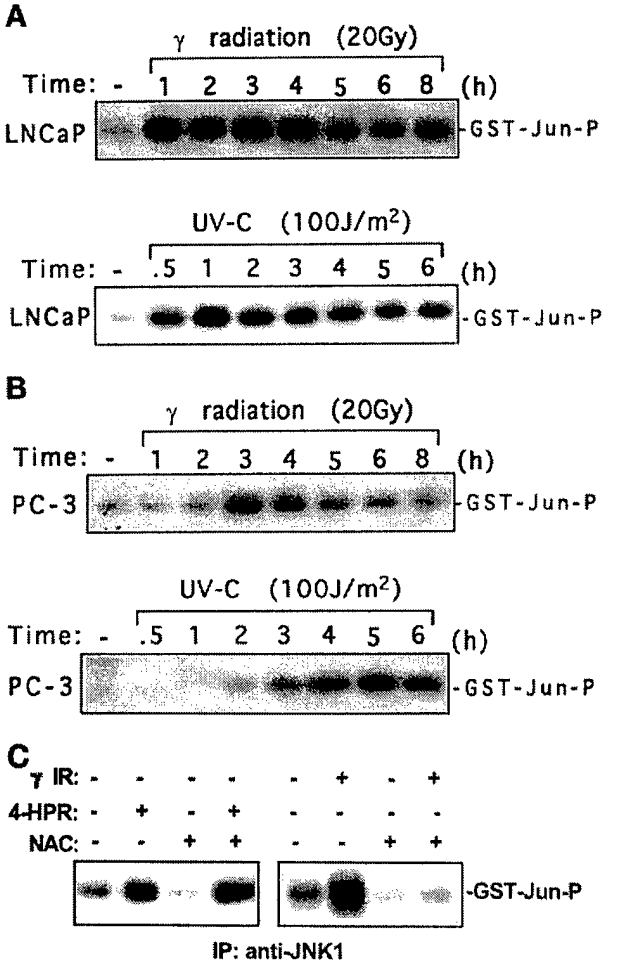


Fig. 5. 4-HPR and γ-radiation induce JNK activation through different mechanisms. LNCaP (A) and PC-3 cells (B) were treated with γ-radiation (20 Gy) or UV-C (100 J/m^2). The cells were harvested at the time points indicated and the endogenous JNK activity was determined by immunocomplex kinase assays as described in *Materials and Methods*. C, LNCaP cells were treated with 4-HPR (20 μM) or γ-radiation (20 Gy) in the presence or absence of NAC (20 mM). Cells were collected 1 h (γ-radiation) or 12 h (4-HPR) after treatment, and endogenous JNK activity was examined by immunocomplex kinase assays.

LNCaP cells than in PC-3 cells (Fig. 6A). Therefore, the difference in susceptibility to 4-HPR in LNCaP and PC-3 cells cannot simply be explained by the expression levels of Bcl-2 and Bcl-X_L. The expression of Bcl-2, but not Bcl-X_L, gradually decreased in LNCaP cells after 4-HPR treatment and became undetectable at the 24-h time point (Fig. 6B). This decrease in Bcl-2 expression occurred after the JNK activation and, therefore, is unlikely to be the cause of JNK induction. The expression levels of Bcl-2 and Bcl-X_L did not change significantly by 4-HPR treatment in PC-3 cells (Fig. 6B).

Activation of JNK Pathway Induces Apoptosis in Both LNCaP and PC-3 Cells. We also tested whether the apoptotic signaling downstream of JNK is intact in PC-3 cells. We transfected an empty vector, HA-tagged JNK1, or Flag-tagged kinase-dead JNK1 mutant (JNK1[APF]) plasmid into LNCaP and PC-3 cells, and studied the induction of apoptosis in the transfected cells. HA-JNK1 has been shown to be activated by forced expression in transfected cells (Yao et al., 1997). The liposome used in the tansfection caused a background of apoptosis (Fig. 7, A and B); however, we observed an increase in apoptosis in the wild-type JNK1-transfected cells compared with cells transfected with the control plasmid or JNK1[APF] (Fig. 7, A and B). These data indicate that both LNCaP and PC-3 cells can undergo apoptosis following induction of the JNK pathway, and that the apoptotic signaling downstream of JNK is functional in PC-3 cells. Because PC-3 is a $p53^{-/-}$ cell line, these results also suggest that p53 is not required for JNK-induced apoptosis.

Interference with JNK Pathway Suppresses 4-HPR-Induced Apoptosis. If JNK activation is required for 4-HPR-induced apoptosis, interference with the JNK pathway should suppress apoptosis induction by 4-HPR. LNCaP cells were treated with 4-HPR in the presence or absence of a chemical that inhibits p38-MAPK (SB202190; Lee et al., 1994) or JNK activation (curcumin; Chen and Tan, 1998). Cotreatment with curcumin suppressed 4-HPR-induced JNK activation and also decreased apoptosis induction (Fig. 8). As expected, because 4-HPR did not induce p38-MAPK activation in LNCaP cells (Fig. 4C), SB202190 failed to affect 4-HPR-induced apoptosis (Fig. 8). SB202190 also did not affect JNK activity at the concentrations tested.

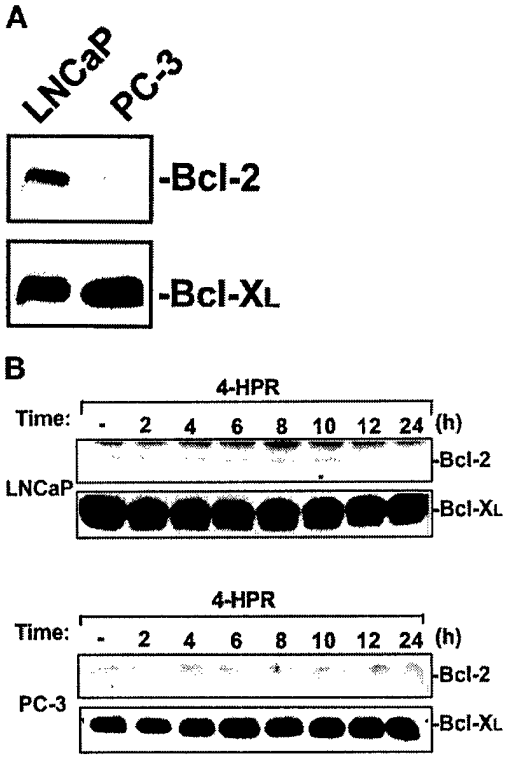


Fig. 6. Bcl-2 expression decreases after 4-HPR treatment in LNCaP cells. A, equal amounts of cell lysate (50 μg) from untreated LNCaP and PC-3 cells were subjected to Western blot analyses for expression of Bcl-2 (exposure time after enhanced chemiluminescence reaction, 5 min) and Bcl-X_L (exposure time, 1 min). B, LNCaP and PC-3 cells were treated with or without 4-HPR (20 μM). At the time points indicated, the cells were harvested and examined by Western blot assays for the expression levels of Bcl-2 (exposure times, 1 min in LNCaP and 3 min in PC-3 cells) and Bcl-X_L (exposure time, 1 min in both LNCaP and PC-3 cells).

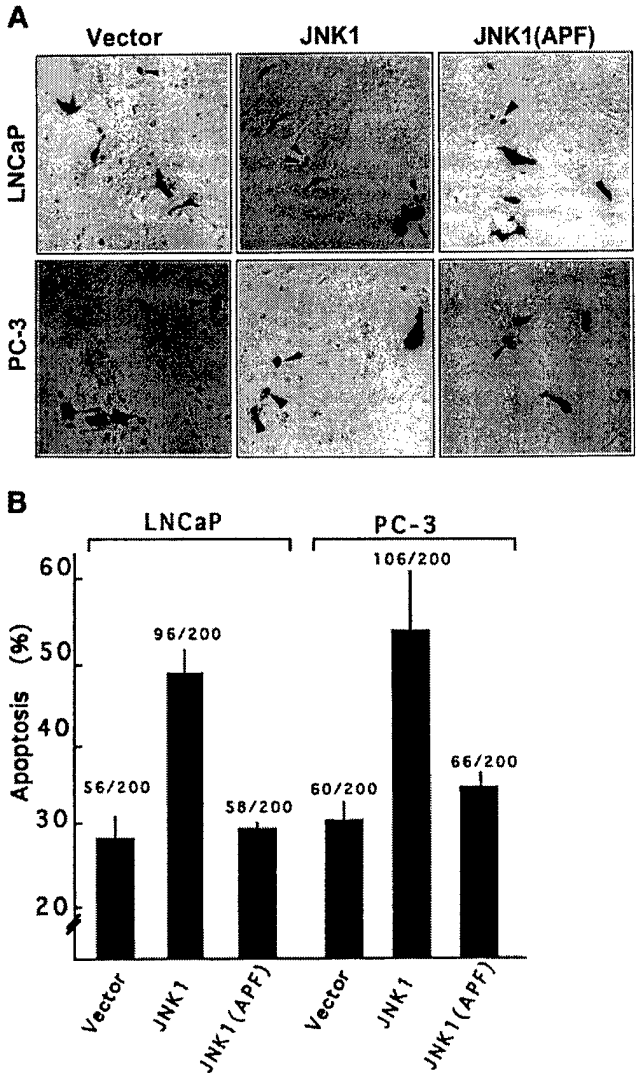


Fig. 7. Forced expression of JNK1 induces apoptosis in both LNCaP and PC-3 cells. LNCaP and PC-3 cells were transfected with plasmids encoding β-galactosidase (3 μg) in combination with the indicated plasmids [HA-JNK1, 2 μg; Flag-JNK1(APF), 2 μg]. The total amount of transfected DNA was normalized with empty vectors. A, cells were stained with X-gal either 24 (PC-3) or 48 h (LNCaP) after transfection. Transfected cells (dark color) with rounding up, shrinking, or membrane-blebbing morphology were identified as apoptotic cells (indicated by arrowheads). B, apoptosis induction was represented as percentage of apoptotic cells per 200 blue cells. Data represent the means ± S.D. of four experiments.

We have not been able to block 4-HPR-induced apoptosis in LNCaP cells by dominant-negative mutants of the JNK pathway in transient transfection-protection assays (data not shown). This was probably because the expression of the mutated kinases was not sufficient to suppress the endogenous kinases in LNCaP cells. HEK293 cells, which allow efficient expression of transfected genes, were used to examine the requirement of the JNK pathway in 4-HPR-induced apoptosis. HEK293 cells were sensitive to 4-HPR-induced JNK activation and apoptosis (Fig. 9, A–C). Interference with the JNK pathway by forced expression of a dominant-negative mutant of JNK1 [JNK(APF)] or SEK1 [SEK1(AL)] suppressed 4-HPR-induced apoptosis (Fig. 9, B and C). The mutated kinase SEK1(AL), which acts immediately upstream of JNK, blocked the activation of cotransfected HA-JNK1 by 4-HPR (Fig. 9D). Collectively, these results suggest that the JNK signaling pathway is important in 4-HPR-induced apoptosis.

Discussion

Our results suggest that the JNK pathway participates in 4-HPR-induced apoptosis. Apoptosis induced by 4-HPR was associated with sustained JNK activation. By forced expression of JNK1, we induced apoptosis in transfected LNCaP and PC-3 cells. Interference with the JNK pathway by dominant-negative kinase mutants suppressed 4-HPR-induced apoptosis in HEK293 cells, suggesting the requirement of the JNK pathway in 4-HPR-induced apoptosis in these cells. Curcumin, an inhibitor of JNK activation, blocked 4-HPR-induced apoptosis in LNCaP cells, suggesting the importance of the JNK signaling. However, the requirement of JNK in 4-HPR-induced apoptosis in LNCaP was not conclusively proved in this study.

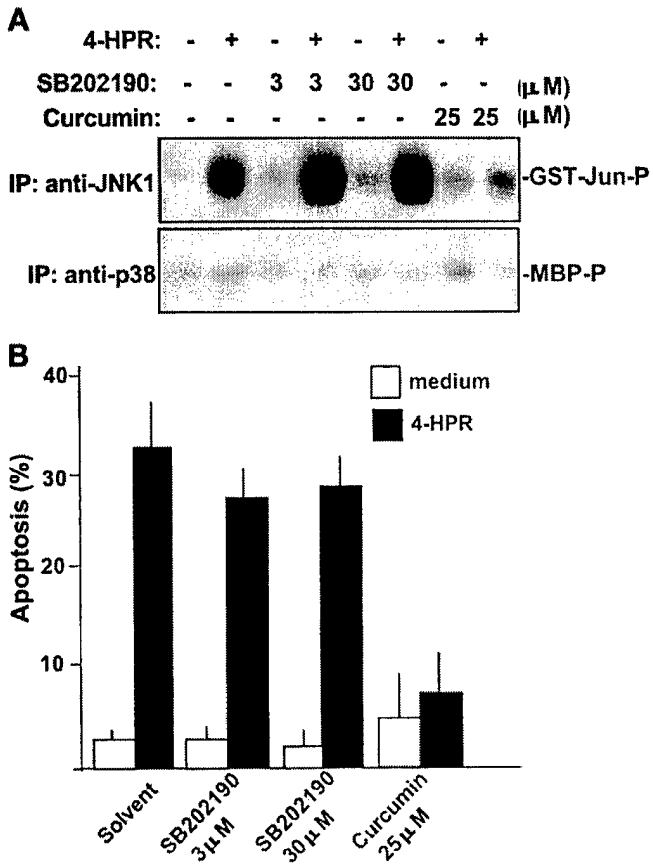


Fig. 8. Interference with the JNK pathway suppresses 4-HPR-induced apoptosis. LNCaP cells were treated with 4-HPR (20 μM) in the presence or absence of SB202190 (30 μM) or curcumin (25 μM). A, cells were collected at the 12-h time point, and endogenous JNK and p38-MAPK activities were examined by immunocomplex kinase assays. B, treated cells were collected at the 30-h time point for nuclear staining. Cells with condensed and fragmented nuclei were identified as apoptotic cells. The results presented are means ± S.D. of three independent experiments.

We found that LNCaP cells were more sensitive to 4-HPR-induced JNK activation and apoptosis than were PC-3 cells. Roberson et al. (1997) reported that 4-HPR is capable of inducing apoptosis in PC-3 cells through a transforming growth factor-β (TGF-β)-dependent pathway. We did observe a growth-inhibitory effect of 4-HPR on PC-3 cells (Fig. 1); however, no significant apoptosis was detected in 4-HPR-treated PC-3 cells by four criteria (flow cytometric analysis, DNA fragmentation, nuclear morphology, and cleavage of caspase 3; Figs. 2 and 3). It is noted that Roberson et al. (1997) used two assays (flow cytometric and DNA fragmentation assays) to detect apoptosis, and they did not compare PC-3 to LNCaP cells. Furthermore, this discrepancy may be due to variations between the different PC-3 lines used in these two studies. One possible variation is in the production of TGF-β or in various components of the TGF-β receptor-signaling pathway. TGF-β has been shown to cause sustained JNK activation (Atfi et al., 1997; Zhou et al., 1999). Whether TGF-β is required for 4-HPR-induced JNK activation is unknown. If that is the case, and TGF-β is required for 4-HPR-induced apoptosis in PC-3 cells as reported (Roberson et al., 1997), defects in TGF-β production or TGF-β receptor signaling may significantly suppress 4-HPR-induced JNK activation and apoptosis. The involvement of TGF-β and the JNK pathway in 4-HPR-induced apoptosis needs to be elucidated by further examination of different cell types. Nevertheless, our data clearly showed the difference in 4-HPR responsiveness between PC-3 and LNCaP cells.

Other genetic factors may result in the differential regulation of JNK and apoptosis by 4-HPR in LNCaP and PC-3 cells. PC-3, an androgen-insensitive, bone marrow-derived, metastasized tumor cell line, is a more progressive prostate carcinoma cell line than the lymph node-derived, androgen-sensitive LNCaP cells (Kaighn et al., 1979; Horoszewicz et al., 1983). Therefore, the failure of PC-3 cells to respond to 4-HPR-induced apoptosis may be due to some defects in the apoptotic-signaling pathway that are not present in LNCaP cells. In addition to androgen unresponsiveness, PC-3 cells have no p53 protein products due to deletions at both p53 alleles (Rubin et al., 1991; Planchon et al., 1995). In contrast, LNCaP cells have wild-type p53 genes. It has been shown that p53 is important for apoptosis induced by γ-radiation and by the adenovirus E1A protein (Debbas and White, 1993; Lowe et al., 1993). It is possible that the lack of p53 protein may contribute to the resistance of PC-3 to apoptosis induction. However, JNK can be activated by radiation in PC-3 cells, although with a slower activation kinetics, in the absence of functional p53 protein. This suggests that p53 is not required for JNK activation. Because JNK phosphorylates both murine and human p53 proteins in vitro (Milne et al., 1995; Alder et al., 1997), it has been suggested that p53 is a downstream effector of the JNK pathway. However, in this

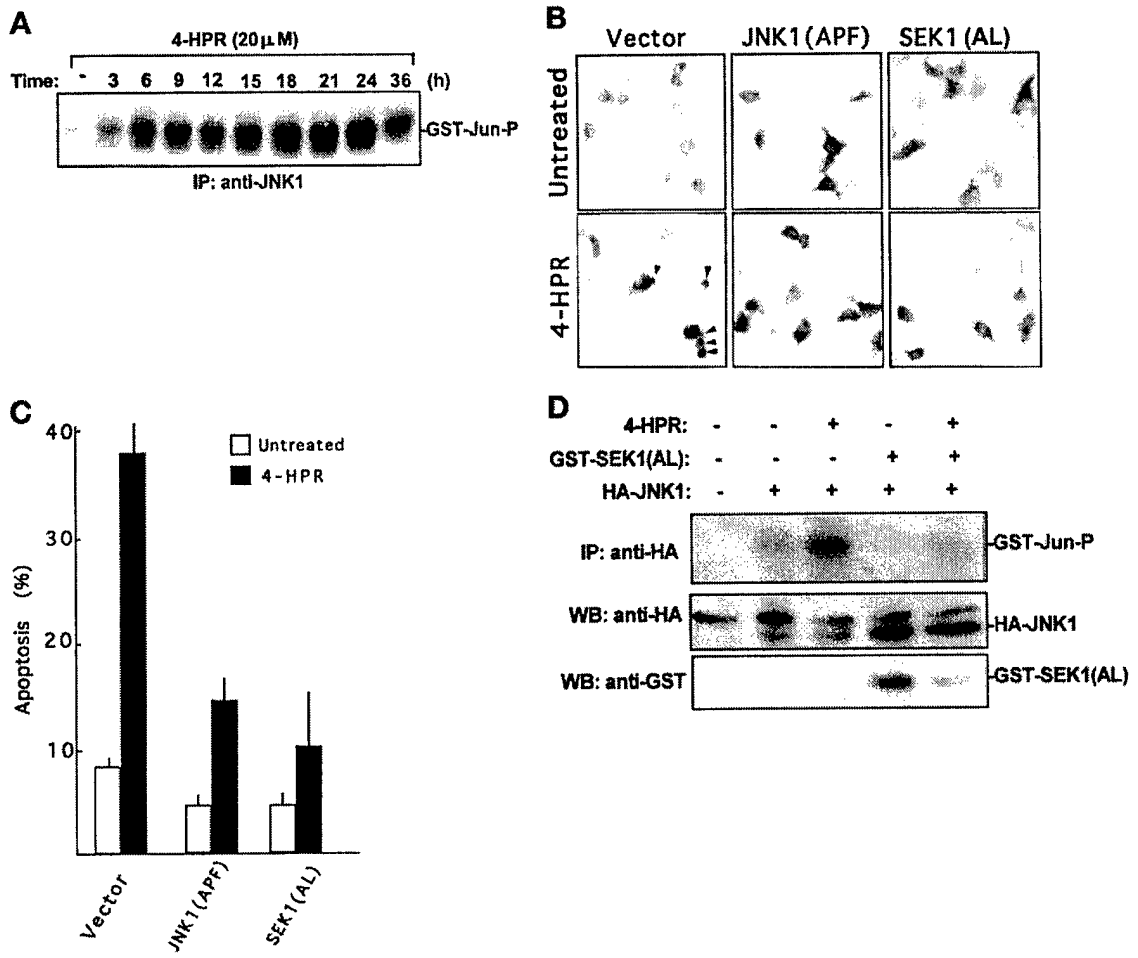


Fig. 9. Expression of dominant-negative mutants in the JNK pathway suppresses 4-HPR-induced apoptosis. A, HEK293 cells were treated with 20 μM 4-HPR; cells were collected at the time points indicated and examined for endogenous JNK activity. B, HEK293 cells were transfected with plasmids encoding β-galactosidase (1 μg) in combination with empty vector or the indicated plasmids encoding dominant-negative kinase mutants (2 μg of each). Transfected cells were cultured in complete medium for 6 h after removing the transfection mixture, treated with or without 4-HPR (20 μM) for 12 h, and then stained with X-gal. Transfected cells (dark color) with rounding up, shrinking, or membrane-blebbing morphology were identified as apoptotic cells (indicated by arrowheads). C, apoptosis induction was represented as percentage of apoptotic cells per 300 blue cells. D, HEK293 cells were transfected with HA-JNK1 plasmid (0.5 μg) plus empty vector (2 μg), or HA-JNK1 (0.5 μg) plus GST-SEK1(AL) (2 μg) plasmids. Cells were treated with or without 4-HPR (20 μM) before harvest. HA-JNK1 activity in the transfected cells was determined by immunocomplex kinase assays. The expressions of the transfected genes were determined by Western blot analyses.

article, we show that forced expression of JNK1 induced apoptosis in the $p53^{-/-}$ PC-3 cells, suggesting that p53 is not required for JNK-mediated apoptosis. Collectively, the data suggest that p53 is not essential for the activation of JNK, and that the p53 protein is not required for the JNK-induced apoptosis. However, this study does not exclude the possibility that p53 may synergize with the JNK pathway to induce apoptosis.

The ability of an antioxidant (NAC) to block γ-radiation-induced JNK activation, but not 4-HPR-induced JNK activation, indicates that these two agents induce JNK through distinct mechanisms. The activation of the JNK pathway by radiation but not by 4-HPR in PC-3 cells shows that genetic alterations in tumor cells may affect one but not other signaling pathways involved in the induction of JNK and apoptosis. Induction of apoptosis in PC-3 cells by forced expression of JNK1 suggests that we may be able to bypass the genetic defects in tumor cells that prevent apoptosis induction by activating JNK directly. Further examination of JNK-mediated apoptotic signaling will be important in the design of more effective cancer therapeutic agents.

Acknowledgments

We thank Drs. R. J. Davis, K.-M. Tchou-Wong, J. R. Woodgett, Z. Yao, and L. I. Zon for their generous gifts; the members of Tan laboratory for their helpful discussions and critical reading of the manuscript; A. Brown, S. Lee, and R. Afshar for technical assistance; and M. Lowe for secretarial assistance.

References

Adams J and Cory S (1998). The Bcl-2 protein family: arbiters of cell survival. *Science (Wash DC)* **281**:1322–1326.

Alder V, Pincus MR, Minamoto T, Fuchs SY, Bluth MJ, Brandt-Rauf PW, Friedman FK, Robinson RC, Chen JM, Wang XW, Harris CC and Ronai Z (1997) Conformation-dependent phosphorylation of p53. *Proc Natl Acad Sci USA* **94**:1686–1691.

Atfi A, Djelloul S, Chastre E, Davis R and Gespach C (1997) Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor β-mediated signaling. *J Biol Chem* **272**:1429–1432.

Chen Y-R, Meyer CF and Tan T-H (1996a) Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in γ radiation-induced apoptosis. *J Biol Chem* **271**:631–634.

Chen Y-R and Tan T-H (1998) Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene* **17:**173–178.

Chen Y-R, Wang W, Kong A-NT and Tan T-H (1998) Molecular mechanisms of c-Jun N-terminal kinase (JNK)-mediates apoptosis induced by anticarcinogenic isothiocyanates. *J Biol Chem* **273:**1769–1775.

Chen Y-R, Wang X, Templeton D, Davis RJ and Tan T-H (1996b) The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and γ radiation: Duration of JNK activation may determine cell death and proliferation. *J Biol Chem* **271:**31929–31936.

Debbas M and White E (1993) Wild-type p53 mediates apoptosis by E1A which is inhibited by E1B. *Genes Dev* **7:**546–554.

Delia D, Aiello A, Lombardi L, Pelicci PG, Grignani F, Grignani F, Formelli F, Menard S, Costa A and Veronesi U (1993) *N*-(4-Hydroxyphenyl)retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid. *Cancer Res* **53:**6036–6041.

Fanjul AN, Delia D, Pierotti MA, Rideout D, Qiu J and Rfahl M (1996) 4-Hydroxyphenyl retinamide is a highly selective activator of retinoid receptors. *J Biol Chem* **271:**22441–22446.

Gupta S, Campbell D, Derijard B and Davis RJ (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science (Wash DC)* **267:**389–393.

Herrmann M, Lorenz H-M, Voll R, Grunke M, Woith W and Kalden JR (1994) A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic Acids Res* **22:**5506–5507.

Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA and Murphy GP (1983) LNCaP model of human prostatic carcinoma. *Cancer Res* **43:**1809–1818.

Ip YT and Davis RJ (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)-from inflammation to development. *Curr Opin Cell Biol* **10:**205–219.

Janknecht R and Hunter T (1997) Activation of the Sap-1a transcription factor by the c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase. *J Biol Chem* **272:**4219–4224.

Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF and Jones LW (1979) Establishment and characterization of a human prostatic carcinoma cell line. *Invest Urol* **17:**16–23.

Kelloff GJ, Crowell JA, Bonne CW, Steele VE, Lubet RA, Greenwald P, Alberts DS, Covey JM, Doody LA and Knapp GG (1994) Clinical development plan: *N*-(4-hydroxylphenyl)retinamide. *J Cell Biochem* **20:**176–196.

Kyriakis JM and Avruch J (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J Biol Chem* **271:**24313–24316.

Lee FS, Hagler J, Chen ZJ and Maniatis T (1997) Activation of the IκBα kinase complex by MEKK1, a kinase for the JNK pathway. *Cell* **88:**213–222.

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, Strickler JE, McLaughlin MM, Siemens IR, Fisher SM, Livi GP, White JR, Adams JL and Young PR (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature (Lond)* **372:**739–746.

Lotan R (1996) Retinoids in cancer chemotherapy. *FASEB J* **10:**1031–1039.

Lowe SW, Schmitt SW, Smith BA, Osborne BA and Jacks T (1993) p53 is required for radiation- induced apoptosis in mouse thymocyte. *Nature (Lond)* **362:**847–849.

Means AL and Gudas LJ (1995) The roles of retinoids in vertebrate development. *Ann Rev Biochem* **64:**201–233.

Meyer CF, Wang X, Chang C and Tan T-H (1996) Interaction between c-Rel and the MEKK1 signaling cascade in mediating κB enhancer activation. *J Biol Chem* **271:**8971–8976.

Milne DM, Campbell LE, Campbell DG and Meek DW (1995) p53 is phosphorylated in vitro and in vivo by an ultraviolet radiation-induced protein kinase characteristic of the c-Jun kinase, JNK1. *J Biol Chem* **270:**5511–5518.

Oridate N, Lotan D, Mitchell MF, Hong WK and Lotan R (1995) Inhibition of proliferation and induction of apoptosis in cervical carcinoma cells by retinoids for chemotherapy. *J Cell Biochem* **23:**80–86.

Park DS, Stefanis L, Irene Yan CY, Farinelli SE and Greene LA (1996) Ordering the cell death pathway. *J Biol Chem* **271:**21898–21905.

Pienta KJ, Nguyen NM and Lehr JE (1993) Treatment of prostate cancer in the rat with the synthetic retinoid fenretinide. *Cancer Res* **53:**224–226.

Planchon SM, Wuerzberger S, Frydma B, Witiak DT, Hitson P, Church DR, Wilding G and Boothman DA (1995) Beta-lapachone-mediated apoptosis in human promyelocytic leukemia (HL-60) and human prostate cancer cells: A p53-independent response. *Cancer Res* **55:**3706–3711.

Pombo CM, Kehrl JH, Irma S, Woodgett JR, Force T and Kyriakis JM (1995) Activation of the SAPK pathway by the human STE20 homologue germinal centre kinase. *Nature (Lond)* **377:**750–754.

Ponzoni M, Bocca P, Chiesa V, Decensi A, Pistoia V, Raffaghello L, Rozzo C and Montaldo PG (1995) Differential effects of *N*-(4-hydroxyphenyl)retinamide and retinoic acid on neuroblastoma cells: Apoptosis versus differentiation. *Cancer Res* **55:**853–861.

Roberson KM, Penland SN, Padilla GM, Selvan RS, Kim C-S, Fine RL and Robertson CN (1997) Fenretinide: induction of apoptosis and endogenous transforming growth factor β in PC-3 prostate cancer cells. *Cell Growth Differ* **8:**101–111.

Rubin S, Hallahan DE, Ashman CR, Brachman DG, Beckett MA, Virudachalam S, Yandell DW and Weichselbaum RR (1991) Two prostate carcinoma cell lines demonstrate abnormalities in tumor suppressor genes. *J Surg Oncol* **46:**31–36.

Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z and Kolesnick RN (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature (Lond)* **380:**75–79.

Whitmarsh AJ and Davis RJ (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med* **74:**589–607.

Whitmarsh AJ, Shore P, Sharrocks AD and Davis RJ (1995) Integration of MAP kinase signal transduction pathways at the serum response element. *Science (Wash DC)* **269:**403–407.

Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinase on apoptosis. *Science (Wash DC)* **270:**1326–1331.

Yao Z, Brown A, Wang XS, Zhou G, Diener K, Zukowski M and Tan T-H (1997) A novel mammalian STE20-related protein kinase, HGK (hematopoietic progenitor kinase/germinal center kinase-like kinase), that specifically activates the JNK/SAPK pathway. *J Biol Chem* **272:**2118–2125.

Zanke BW, Boudreau K, Rubie E, Winnett E, Tibbles LA, Zon L, Kyriakis J, Liu F-F and Woodgett JR (1996) The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr Biol* **6:**606–613.

Zhou G, Lee SC, Yao Z and Tan T-H (1999) Hematopoietic progenitor kinase 1 is a component of tansforming gowth factor β-induced c-Jun N-terminal kinase signaling cascade. *J Biol Chem* **274:**13133–13138.

Send reprint requests to: Dr. Tse-Hua Tan, Department of Microbiology and Immunology, Baylor College of Medicine, M929, One Baylor Plaza, Houston, TX 77030. E-mail: ttan@bcm.tmc.edu

Oncogene (1999) 18, 7370–7377

http://www.stockton-press.co.uk/onc

Caspase-mediated cleavage and functional changes of hematopoietic progenitor kinase 1 (HPK1)

Yi-Rong Chen[1], Christian F Meyer[1], Bushra Ahmed[1], Zhengbin Yao[2] and Tse-Hua Tan*[,1]

[1]*Department of Microbiology and Immunology, Baylor College of Medicine, M929, One Baylor Plaza, Houston, Texas 77030, USA;* [2]*CNS Department, Hoechst Marion Roussel, Bridgewater, New Jersey, NJ 08807, USA*

Activation of c-Jun N-terminal kinase (JNK) by Fas ligation is caspase-dependent, suggesting that caspases may regulate activators of the JNK pathway. Here, we report that an upstream activator of JNK, hematopoietic progenitor kinase 1 (HPK1), was cleaved during apoptosis. Cleavage of HPK1 was blocked by peptide inhibitors for caspases. HPK1 was efficiently processed by recombinant caspase 3 *in vitro*. A conserved caspase recognition site, DDVD (amino acids 382–385), was found in the HPK1 protein sequence. By testing HPK1 proteins with *in vivo* and *in vitro* cleavage assays, we showed that aspartic acid residue 385 is the target for caspases. HPK1 cleavage separated the amino N-terminal kinase domain from the carboxyl C-terminal regulatory domain, and enhanced HPK1 kinase activity. Unlike the full-length HPK1, the N-terminal cleaved product failed to bind adaptor molecules Grb2 (growth factor receptor-bound protein 2) and Crk (CT10 regulator of kinase). The C-terminal fragment, although having three proline-rich domains, bound to Grb2 and Crk less efficiently than the full-length HPK1 protein. Taken together, the cleavage of HPK1 by caspase profoundly changed its biochemical properties.

Keywords: HPK1; JNK; caspase; apoptosis; adaptor

Introduction

Apoptosis is important in regulating development and maintaining homeostasis in multicellular organisms (reviewed in Steller, 1995; Thompson, 1995). Apoptosis is positively and negatively regulated by genetic and biochemical programs (reviewed in Steller, 1995). In recent years, the molecular mechanisms of apoptosis have gradually been unfolded. Caspases, aspartate-directed cysteine proteases, are required for apoptosis. The blockage of caspase activation by peptide inhibitors or by viral proteins, such as the pox virus protein CrmA or baculovirus p35, suppresses apoptosis progression (reviewed in Salvesen and Dixit, 1997). Cleavage by caspases may enhance the biochemical activity of their substrates, e.g., caspases themselves (Salvesen and Dixit, 1997) and protein kinase C δ (Emoto *et al.*, 1995). Cleavage by caspases can also diminish normal functions of the substrates, such as poly-(ADP-ribose) polymerase (PARP) (Lazebnik *et al.*, 1994), DNA-dependent protein kinase (Song *et al.*, 1996), MDM2 (Erhardt *et al.*, 1997), $p21^{Cip1/Waf1}$, and $p27^{Kip1}$ (Levkau *et al.*, 1998). In addition, cleavage of nuclear lamin (Lazebnik *et al.*, 1995), gelsolin (Kothakota *et al.*, 1997), and focal adhesion kinase (FAK) (Wen *et al.*, 1997) by caspases is involved in the morphological changes found in apoptotic cells.

The c-Jun N-terminal kinase (JNK) family, also called stress-activated protein kinase (SAPK), belongs to the mitogen-activated protein kinase (MAPK) superfamily (reviewed in Ip and Davis, 1998). JNK is activated in apoptosis induced by various stimuli (reviewed in Ip and Davis, 1998). Activation of the JNK pathway can lead to cell death (Xia *et al.*, 1995; Chen *et al.*, 1996b; Ichijo *et al.*, 1997). Interference with the JNK pathway by dominant-negative kinases or an antisense to JNK suppresses apoptosis (Xia *et al.*, 1995; Chen *et al.*, 1996b, 1998; Verheij *et al.*, 1996; Zanke *et al.*, 1996; Goillot *et al.*, 1997; Seimiya *et al.*, 1997; Kasibhatla *et al.*, 1998). However, reports on the importance of JNK in Fas-mediated apoptosis were controversial (Brenner *et al.*, 1997; Goillot *et al.*, 1997; Lenczowski *et al.*, 1997). The exact mechanism by which JNK was activated by apoptotic stimuli was still unclear. Previously, we and others have shown that JNK can be activated by oxidative stresses induced by different apoptotic stimuli, and that this JNK activation is independent of caspase activation (Park *et al.*, 1996; Chen *et al.*, 1998). Nevertheless, in Fas-mediated apoptosis, JNK induction is suppressed by a caspase inhibitor (Chen *et al.*, 1996b; Lenczowski *et al.*, 1997), indicating that certain JNK activator(s) may be regulated by caspases.

Like other MAP kinases, JNK is regulated by a kinase cascade, MAPK kinase kinase (MAP3K/MEKK)→MAPK kinase (MAP2K/MKK)→MAPK (JNK) (Kyriakis and Avruch, 1996). To date, two MAP2K/MKKs, MKK4/SEK1 and MKK7, are known to activate JNK preferentially (Tournier *et al.*, 1997; Yao *et al.*, 1997b). Multiple MAP3K/MEKKs were found to activate the JNK pathway via MKK4/SEK1 or MKK7. These MEKK-like kinases include MEKK1–4, MAPKKK5/ASK1, TAK1, Tpl-2/Cot, MLK3/SPRK, and MUK/DLK (reviewed in Fanger *et al.*, 1997). In addition, several yeast STE20-like kinases, such as p21-GTPase (Rac1/Cdc42)-activated kinases (PAKs) and germinal center kinase (GCK), were also shown to activate the JNK pathway (Bagrodia *et al.*, 1995; Pombo *et al.*, 1995). We and others have cloned and characterized the hematopoietic progenitor kinase 1 (HPK1) (Hu *et al.*, 1996; Kiefer *et al.*, 1996), which is homologous to GCK. Our results indicate that HPK1 regulates the JNK pathway through the HPK1 → *MEKK1*, *TAK1* → *MKK4/SEK*, *MKK7* → *JNK* cascade (Hu *et al.*, 1996; Wang *et al.*, 1997; Zhou *et al.*, 1999). HPK1 may mediate extracellular signals

*Correspondence: T-H Tan
Received 16 April 1999; revised 22 July 1999; accepted 5 August 1999

through interaction with Src-homology 3 (SH3) domain-containing adaptor molecules (Anafi *et al.*, 1997; Oehrl *et al.*, 1998; Ling *et al.*, 1999). Here we report that HPK1 was cleaved by caspase activity during apoptosis. This cleavage separated HPK1's kinase domain from its C-terminal regulatory domain, and enhanced its kinase activity. Cleavage of HPK1 by caspases also greatly reduced its ability to associate with adaptor molecules Grb2 and Crk.

Results

Caspase-mediated cleavage of HPK1 during Fas ligation-induced apoptosis

Our previous finding shows that both γ radiation and anti-Fas treatment induce persistent JNK activation in Jurkat cells, but the mechanisms of JNK induction in these two events are different. Fas-mediated JNK activation was suppressed by a caspase inhibitor, z-VAD-FK. In contrast, γ radiation-induced JNK activation was not suppressed by z-VAD-FK (Figure 1). The suppression of Fas-mediated JNK activation by a caspase inhibitor suggests that the JNK pathway can be activated by a caspase-dependent mechanism.

HPK1 is a JNK upstream activator, which regulates JNK through the HPK1→MEKK1, TAK1→MKK4, MKK7→JNK pathway (Hu *et al.*, 1996; Wang *et al.*, 1997). By examining the protein sequence of human HPK1, we located one consensus caspase target site between the first and second proline-rich motifs (Figure 2a). This sequence, DDVD (amino acids 382–385), is homologous to the substrate sequence DEXD (X stands for any amino acid) for a group of caspases, including caspases 2, 3, and 7 (Salvesen and Dixit, 1997). Processing of HPK1 at this site would separate the N-terminal kinase domain from the C-terminal domain, with predicted molecular weights of 43 kD and 54 kD, respectively (Figure 2a).

To examine the possible processing of HPK1 during apoptosis, Jurkat human T cells were treated with an anti-Fas antibody (CH-11), and endogenous HPK1 in treated cells was examined by Western blotting using an anti-N-terminal HPK1 antibody (Figure 2a,b). We found that a new protein band with an apparent

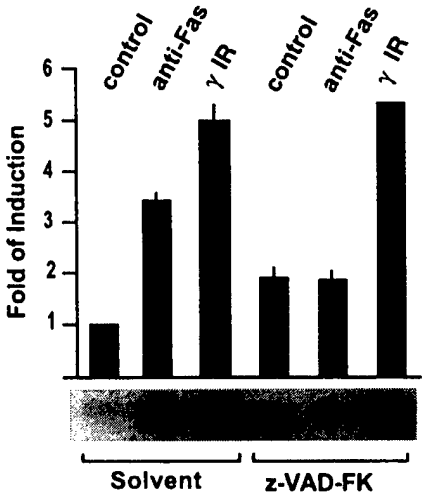


Figure 1 Fas-mediated, but not γ radiation-induced, JNK activation is caspase dependent. Jurkat T cells were preincubated with z-VAD-FK (100 μM) for 2 h, and then treated with either γ radiation (100 Gy) or Fas ligation (CH-11, 100 ng/ml) for 3 h. The cells were harvested and endogenous JNK activity was examined by immunocomplex kinase assays using an anti-JNK1 antibody. The results of the kinase assays were quantitated by a densitimeter, data presented are the means and standard deviations of three measurements

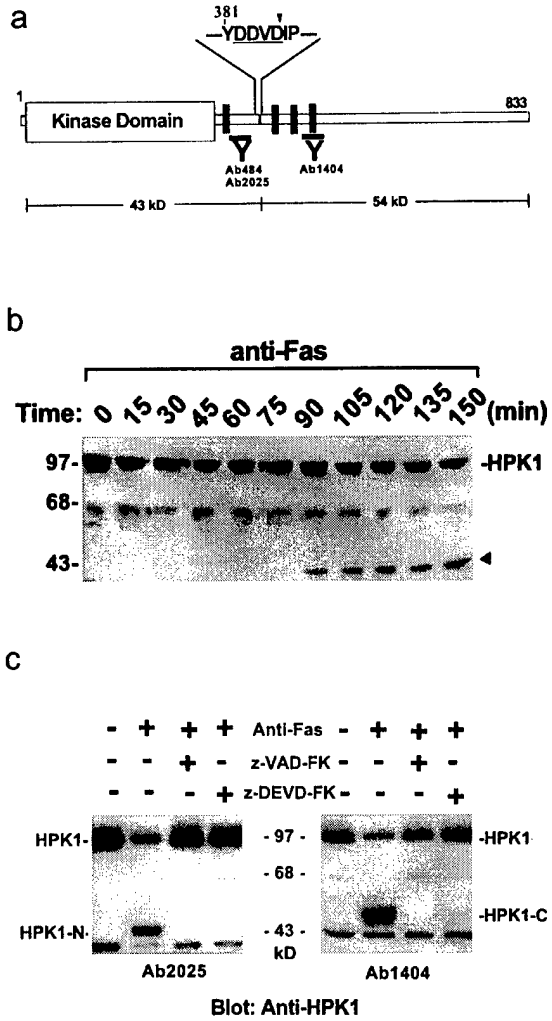


Figure 2 Caspase-mediated HPK1 cleavage during Fas ligation-induced apoptosis. (**a**) A consensus recognition site (underlined) for caspases was found in the human HPK1 protein sequence. The triangle indicates the putative caspase cleavage site. The regions recognized by three different anti-HPK1 antibodies (Ab484, Ab2025, and Ab1404) are shown. Black bars represent proline-rich motifs. (**b**) Jurkat cells were treated with anti-Fas (CH-11, 100 ng/ml), and were collected at different time points as indicated. Endogenous HPK1 levels were analysed by SDS–PAGE and Western blotting using an anti-HPK1 antibody (Ab2025). (**c**) Jurkat cells were treated with anti-Fas in the presence or absence of the caspase inhibitors z-VAD-FK (100 μM) or z-DEVD-FK (50 μM). Endogenous HPK1 proteins were analysed by SDS–PAGE and Western blotting using an anti-N-terminus antibody (Ab2025) or an anti-C-terminus antibody (Ab1404)

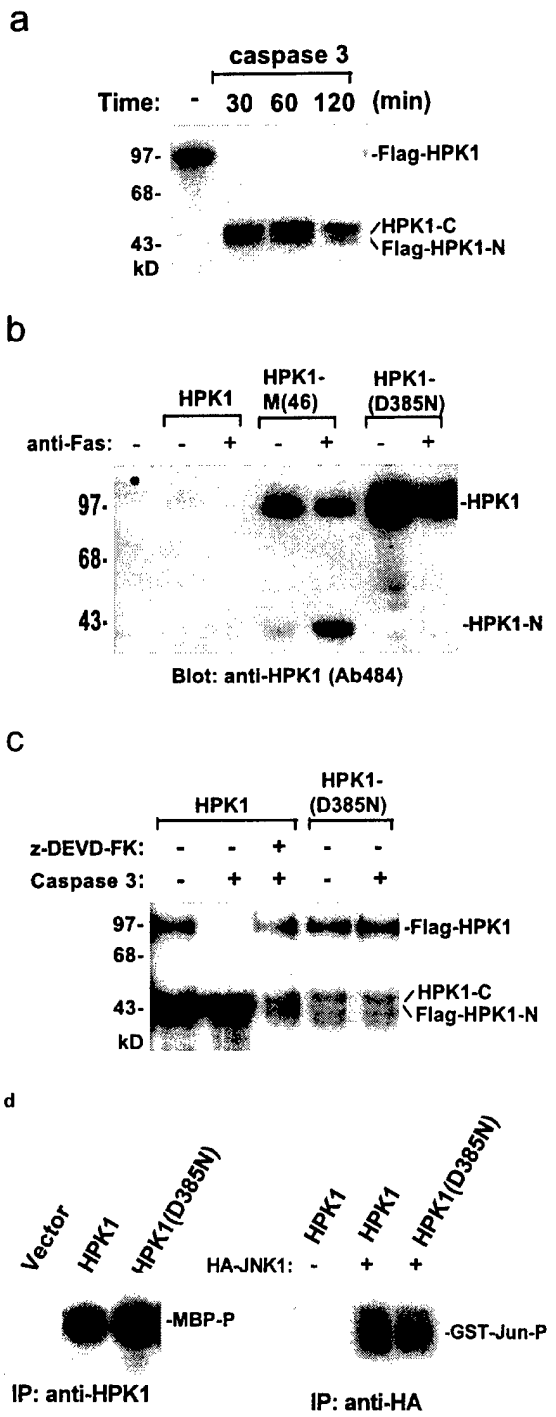


Figure 3 Aspartic acid residue 385 is the caspase cleavage site in HPK1 (**a**) *in vitro* translated ^{35}S-labeled Flag-tagged HPK1 proteins were immunoprecipitated from reticulocyte lysate using an anti-Flag antibody (M2), and incubated with recombinant caspase 3 for indicated times. Reaction mixtures were resolved by SDS–PAGE and analysed by autoradiography. (**b**) HeLa cells were transfected with various HPK1 constructs for 8 h. Transfected cells were incubated in complete medium for 12–14 h, then treated with UV-C (30 J/m^2). Irradiated cells were then incubated with or without an anti-Fas antibody (CH-11, 100 ng/ml) for 4 h. Expression of HPK1 proteins was examined by Western blotting using an anti-HPK1 antibody (Ab484). (**c**) *in vitro* translated ^{35}S-labeled Flag-tagged HPK1 proteins were immunoprecipitated from reticulocyte lysate using an anti-Flag antibody (M2), and incubated with caspase 3 for 3 h in the presence or absence of z-DEVD-FK (50 nM). Reaction mixtures were resolved by SDS–PAGE and analysed by autoradiography. (**d**) Left panel: 293 cells were transfected with empty vector, HPK1, or HPK1-(D385) expressing plasmid (1 μg each). Right panel: 293 cells were transfected with different combinations of expressing plasmids including empty vector plus HPK1 (1 μg each), HPK1 plus HA-JNK1 (1 μg each), or HPK1-(D385N) plus HA-JNK1 (1 μg each). HPK1 and HA-JNK1 activities in the transfected cells were determined by immunocomplex assays as described in Materials and methods

molecular weight of 43 kD appeared on the Western blot (Figure 2b), suggesting HPK1 is cleaved during apoptosis. To verify if the HPK1 cleavage was mediated by caspases, Jurkat T cells were treated with the anti-Fas antibody in the presence or absence of a pan-caspase inhibitor, z-VAD-FK or a selective caspase inhibitor, z-DEVD-FK. The treated cell lysates were then analysed by Western blotting with antibodies against either the N-terminus (Ab2025) or the C-terminus (Ab1404) of HPK1 (Figure 2c). Both anti-HPK1 antibodies recognized one new protein band in anti-Fas-treated cells, which was absent in untreated cells. These protein bands had molecular weights corresponding to the predicted cleavage products (Figure 2a,c), suggesting that HPK1 was processed at the putative cleavage site. Furthermore, the HPK1 cleavage was blocked by co-treatment of the cells with either caspase inhibitor (Figure 2c), suggesting that the cleavage was mediated through a caspase-dependent pathway.

Aspartic acid residue 385 is the caspase cleavage site in HPK1

Sequence examination and the Western blot analysis of the cleaved HPK1 proteins suggested that DDVD^{385} is a possible caspase recognition site in HPK1. Inhibition of HPK1 cleavage by the caspase inhibitor, z-DEVD-FK, also suggests that HPK1 cleavage is dependent on a DEVD-oriented caspase (or caspases) such as caspase 2, 3 and/or 7. To verify that HPK1 is a direct target for caspases, the *in vitro* translated ^{35}S-methionine-labeled HPK1 protein was used as a substrate for caspase assays. Recombinant caspase 3, which has a DEVD-oriented substrate specificity (Salvesen and Dixit, 1997), was used in the assays. Caspase 3 processed HPK1 efficiently, since the full-length HPK1 proteins were almost completely cleaved in 30 min (Figure 3a). This result indicates that HPK1 is a direct substrate for caspase 3, and suggests that HPK1 may be cleaved by caspase-3 like proteases *in vivo*.

Our data suggest that the DDVD^{385} motif in HPK1 is a potential target for caspases. Also, we found an identical DDVD motif (amino acids 381–384) in murine HPK1 protein sequence (Kiefer *et al*., 1996), suggesting that this motif may be a conserved caspase cleavage site. To examine whether DDVD^{385} is the real caspase recognition site, we generated an HPK1 mutant by changing the critical aspartic residue (D385) to an asparagine (N). The susceptibility of the HPK1-(D385N) protein to caspases was tested by an *in vivo* system. HeLa cells were transfected with different HPK1 constructs. The transfected cells were irradiated with a low dose of UV-C (30 J/m^2) to sensitize them to

Fas ligation (Rehemtulla *et al.*, 1997), and were then incubated with or without an anti-Fas antibody. The expression and processing of the transfected HPK1 were examined by Western blot analyses using an anti-HPK1 (N-terminus) antibody (Figure 3b). Wild-type HPK1 was not expressed well in HeLa cells, but the kinase-dead HPK1-M(46) was expressed efficiently, and the level of the N-terminal cleaved fragment (HPK1-N) increased after Fas ligation (Figure 3b), indicating that HPK1-M(46) was processed by caspases. The N-terminal cleaved fragment was not detected in cells transfected with HPK1-(D385N) after Fas ligation, suggesting that the mutated protein was resistant to caspase cleavage. This result also suggests that the sequence, $DDVD^{385}$, is the major target of caspases in a cellular context.

In vitro transcribed, translated, and ^{35}S-methionine-labeled HPK1 proteins were also tested with an *in vitro* cleavage assay using recombinant caspase 3 proteins. The wild-type HPK1 protein was cleaved by caspase 3, and the cleavage was inhibited by z-DEVD-FK, indicating that the cleavage is mainly caused by caspase 3, but not by contaminated proteases from reticulocyte lysate. In contrast, HPK1-(D385N) was completely resistant to caspase 3, even after a 3 h incubation (Figure 3c). This result is consistent with the *in vivo* experiments (Figure 3b), suggesting that aspartic acid 385 is the target for caspases in the HPK1 protein. The HPK1-(D385N) mutant showed similar kinase activity and JNK-activating ability as the wild-type HPK1 (Figure 3d), indicating that the point mutation only affected HPK1's susceptibility to caspases but had no other global effect on HPK1.

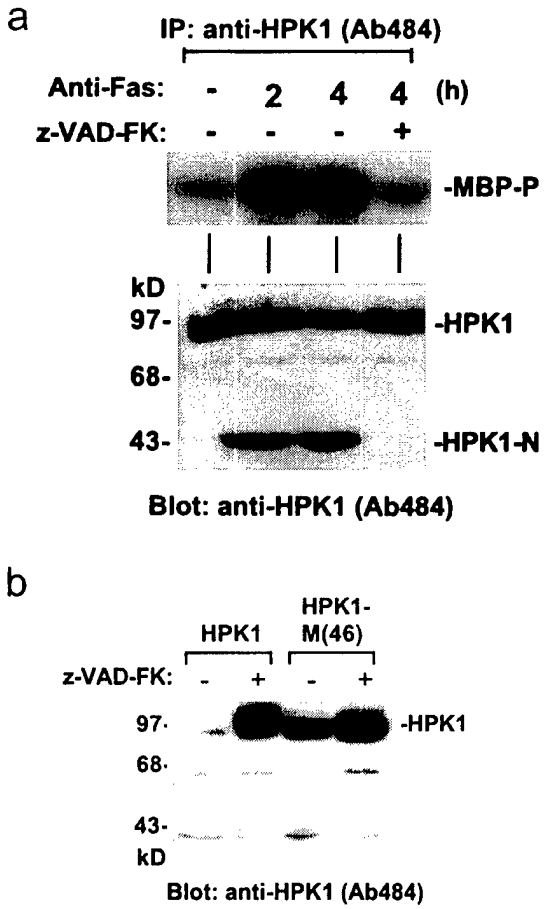


Figure 4 A potential reciprocal regulation between HPK1 and caspases. (**a**) Jurkat cells were treated with anti-Fas (CH-11, 100 ng/ml) in the presence or absence of z-VAD-FK (100 μM). Endogenous HPK1 proteins were analysed by SDS–PAGE and Western blotting using an anti-N-terminal HPK1 antibody (Ab484). HPK1 activity was isolated with Ab484 and examined by immunocomplex kinase assays using MBP as a substrate. (**b**) HeLa cells were transfected with a plasmid encoding HPK1 or HPK1-M(46). Transfected cells were then incubated in complete medium with or without z-VAD-FK (100 μM) for 24 h. Cells were collected and the expression of HPK1 proteins was examined by Western blotting using an anti-HPK1 antibody (Ab484)

A potential reciprocal regulation between HPK1 and caspases

Because the HPK1 cleavage separated the kinase domain from the C-terminal regulatory domain, we examined whether the cleavage of HPK1 affects its kinase activity. HPK1 was immunoprecipitated from untreated or anti-Fas-treated Jurkat cells by an antibody (Ab484) that recognized the N-terminus of HPK1 proteins (Figure 4a), and then examined by *in vitro* kinase assays using myelin basic protein (MBP) as a substrate. HPK1 activity increased in anti-Fas treated cells when compared with untreated cells (Figure 4a). This activation was suppressed in the presence of the caspase inhibitor z-VAD-FK during Fas ligation, suggesting that the cleavage of HPK1 by caspases enhances its kinase activity.

It is interesting to note that the expression level of wild-type HPK1 was much less than that of kinase-dead HPK1-M(46) in transfected HeLa cells (Figure 3b). One possibility is that overexpression of HPK1 may activate the JNK pathway and downstream apoptotic signaling, subsequently, induce apoptosis in HeLa cells. Therefore, wild-type HPK1 may either induce its own cleavage or stop its own expression by inducing caspase activation and apoptosis. To test this possibility, we expressed both HPK1 and HPK1-M(46) in HeLa cells in the presence or absence of the caspase inhibitor, z-VAD-FK. The addition of the caspase inhibitor greatly increased the expression level of full-length HPK1, while only marginally increased the HPK1-M(46) levels (Figure 4b). This result suggests that HPK1's kinase activity induced caspase activation and its own degradation.

The N-terminal HPK1 fragment retains JNK activating ability

Since HPK1 is an upstream JNK activator and the JNK pathway is involved in apoptotic signaling, we then examined whether cleavage of HPK1 affects its ability to induce JNK activation. Co-expression of HPK and JNK enhanced JNK kinase activity (Figure 5). HPK1-N(1-385), corresponding to the N-terminal HPK1 cleaved product, induced stronger JNK activation than the full-length HPK1 did (Figure 5). The kinase-dead HPK1-M(46) and C-terminal fragment HPK1-C(386-833) failed to activate JNK (Figure 5). This result suggests that cleavage of HPK1 may enhance its JNK activating ability.

Cleavage of HPK1 reduces its interaction with adaptor molecules

We and others found that HPK1 physically interacts with adaptor molecules including Crk, CrkL, and Grb2 via proline-rich motifs in the C-terminal regulatory region (Anafi *et al.*, 1997; Oehrl *et al.*, 1998; Ling *et al.*, 1999). Cleavage of HPK1 by caspases separates the N-terminus of HPK1 (containing kinase domain and the first proline-rich motif) from the C-terminus (containing the remaining three proline-rich motifs (Figure 2a). Therefore, the cleavage of HPK1 may affect its ability to associate with adaptor molecules and, very likely, change HPK1 localization and functions. We tested whether cleaved HPK1 fragments bind to adaptor molecules Grb2 and Crk. Cell lysates isolated from Jurkat cells treated with or without anti-Fas were incubated with GST-Crk (or GST-Grb2) fusion proteins, and then were affinity-purified by GSH-sepharose beads followed by repeated washes. The precipitated complexes were subjected to SDS–PAGE and Western blot analyses using anti-HPK1 antibodies (anti-N-terminus Ab484 and anti-C-terminus Ab1404). The full-length HPK1 in either untreated or anti-Fas treated cell lysate bound to GST-Crk and GST-Grb2 proteins. The N-terminal cleaved fragment failed to bind GST-Crk and GST-Grb2 (Figure 6a). The C-terminal cleaved fragment of HPK1 retained the Crk (or Grb2)-binding property (Figure 6b); however, its binding to Crk (or Grb2) was much less efficient than the full-length HPK1 protein (Figure 6b).

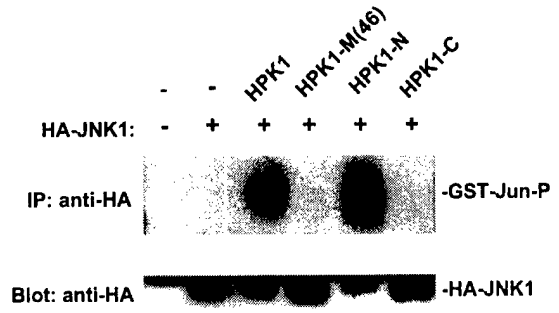


Figure 5 N-terminal HPK1 fragment retains JNK activating ability. 293 cells were transfected with HA-JNK1 (1 μg) in combination with indicated plasmids (1 μg each). The total amount of DNA was normalized with empty vectors. Thirty-six hours after removing the transfection mixture, cell lysates were prepared and JNK activity was immunoprecipitated by an anti-HA antibody (12CA5) and examined by *in vitro* kinase assays. Expression of HA-JNK was examined by Western blotting using an anti-HA antibody

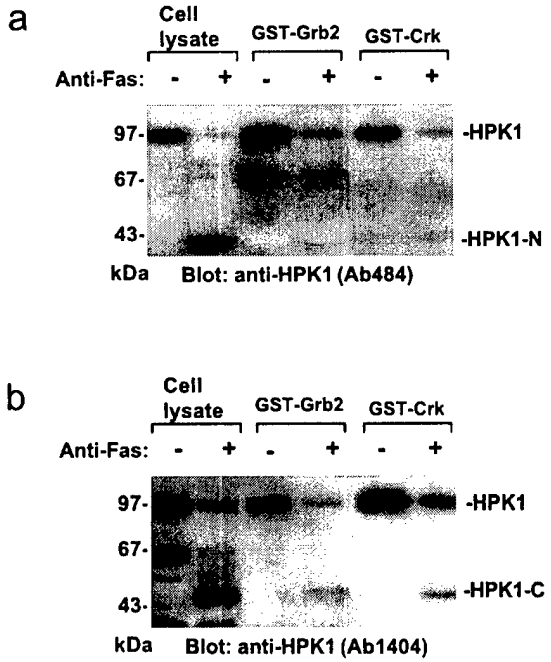


Figure 6 Decreases in HPK1-adaptor molecules interaction after caspase cleavage. (**a** and **b**) Cell lysates (500 μg) isolated from Jurkat cells treated with or without anti-Fas (CH-11, 100 ng/ml; 4 h) were incubated with GST-Grb2 (or GST-Crk) fusion proteins (20 μg). The protein complexes were affinity-purified by GSH-sepharose beads followed by repeated washes as described in Materials and methods. The precipitated complexes were subjected to SDS–PAGE and Western blot analyses using anti-HPK1 antibodies (**a**, anti-N-terminus Ab484; **b**, anti-C-terminus Ab1404). Crude cell lysate (50 μg) from untreated and anti-Fas treated cells were used as controls

Discussion

Both caspases and the JNK pathway participate in apoptotic signaling induced by various stimuli. However, the relative order of JNK and caspases in apoptotic signaling differ in apoptosis induced by distinct stimuli. In apoptosis induced by growth factor withdrawal, radiation, or anti-cancer drugs, JNK activation occurs in the absence of caspase activity (Chen *et al.*, 1996b, 1998; Park *et al.*, 1996). In contrast, caspase activity is required for JNK activation in Fas-mediated apoptosis (Chen *et al.*, 1996b; Lenczowski *et al.*, 1997). The importance of the JNK pathway in Fas-mediated apoptosis is controversial (Brenner *et al.*, 1997; Goillot *et al.*, 1997; Lenczowski *et al.*, 1997).

Recently, several JNK upstream activators including MEKK1, PAK2/hPAK65, and Mst1 were shown to be substrates of caspases (Cardone *et al.*, 1997; Rudel and Bokoch, 1997; Deak *et al.*, 1998; Graves *et al.*, 1998; Widmann *et al.*, 1998). The similarities among these reports are that the cleavage separates the kinase domain from the regulatory domain, and enhances the kinase activity (Cardone *et al.*, 1997; Rudel and Bokoch, 1997; Deak *et al.*, 1998; Graves *et al.*, 1998; Widmann *et al.*, 1998). The cleaved kinases have either the same or enhanced ability to activate JNK. In this report, we show that HPK1 was also cleaved by caspases in Fas-mediated apoptosis. The cleavage of HPK1 separated the kinase domain from the C-terminal regulatory domain, enhanced its kinase activity, and may enhance downstream JNK activation. We were unable to block Fas-mediated JNK activation by kinase-dead HPK1 mutants (data not shown). One possibility is that in the presence of cleavage and activation of multiple JNK upstream kinases by caspases, blocking of HPK1 alone is not sufficient to suppress JNK activation. In addition to HPK1, we found that other two HPK1-like, JNK activating kinases, GCK-like kinase (GLK) and HPK1/GCK homologous kinase (HGK) (Diener *et*

al., 1997; Yao et al., 1997a), were also cleaved by caspase activity during Fas-mediated apoptosis (data not shown). Taken together, our and others' data suggest that the cleavage of JNK upstream regulators is a conserved mechanism, which may mediate JNK activation and other downstream effects. It has been shown that JNK activation is required for caspase activation by certain stimuli (Seimiya et al., 1997; Shiah et al., 1998). It is possible that apoptotic signaling is a circuit that death stimuli can enter at either the JNK pathway or the caspase cascade, and the signaling circuit may amplify the death signal through the reciprocal interaction between these two signaling modules.

How the cleavage of HPK1 enhances its kinase activity is intriguing. The regulatory domain of *S. pombe* PAK1 has been shown to inhibit its own kinase activity, and the binding of Cdc42 GTPase releases the inhibitory effect. We found that the HPK1 kinase domain construct is more active than the full-length protein, suggesting that the C-terminus of HPK1 may also serve as an inhibitory domain. Cleavage of HPK1 may relieve this self-inhibitory effect. One interesting structural feature of HPK1 is its four proline-rich motifs (putative SH3 domain-binding sites) in its regulatory region (Hu et al., 1996). We and others found that HPK1 physically interacts with adaptor molecules including Crk, CrkL, and Grb2 via these proline-rich motifs (Anafi et al., 1997; Oehrl et al., 1998; Ling et al., 1999). Co-expression of Crk with CrkL enhances HPK1 activity through mechanisms yet unknown (Ling et al., 1999). It is possible that binding of adaptors to the proline-rich motifs of HPK1 also relieves the self-inhibitory effect.

Cleavage of HPK1 by caspases separated the N-terminus of HPK1 (containing the first proline-rich domain) from the C-terminus (containing proline-rich domains 2, 3 and 4), and decreased its binding to adaptor molecules Grb2 and Crk. It has been shown that interaction between HPK1 and adaptor molecules may play a role in recruiting HPK1 to the surface receptor complexes (Anafi et al., 1997; Ling et al., 1999). The cleavage of HPK1 greatly reduced its ability to associate with Grb2 and Crk and, very likely, may change HPK1 localization and functions. Previously, we also observed that HPK1 mutants interfere with IL-2 promoter activation induced by T-cell activation signals (Ling et al., 1999). This result suggests that HPK1 may mediate T-cell receptor signaling. Our data indicate that cleaved HPK1 is still capable of activating the JNK pathway; however, whether and how the cleavage will affect HPK1's other downstream functions remains uncertain. It will be important to examine whether cleavage of HPK1 disables other T-cell activation signaling but specifically leaves the JNK pathway in activated condition. If that is the case, it will be a novel mechanism that caspases inhibit mitogenic signaling and, at the same time, leave the apoptotic signaling intact.

Materials and methods

Cells, antibodies, and reagents

Human Jurkat T cells (clone J.LEI) were cultured as described (Chen et al., 1996a). HeLa cells and human embryonic kidney 293 cells were cultured in DMEM supplemented with 10% fetal calf serum and streptomycin/penicillin. Rabbit anti-JNK1 antibody (Ab101) and anti-HPK1 antibodies (Ab2025, Ab484, and Ab1404) were described previously (Chen et al., 1996b; Hu et al., 1996; Ling et al., 1999). The horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody was obtained from Sigma. The anti-Flag (M2) and anti-HA (12CA5) antibodies were purchased from Kodak and Boehringer Mannheim, respectively. Protein A and G-agarose beads were purchased from Bio-Rad and Santa Cruz, respectively. Caspase inhibitors, z-VAD-FK and z-DEVD-FK, and the anti-Fas antibody (CH-11) were purchased from Kamiya Biomedical. Myelin basic protein (MBP) was purchased from Gibco. Caspase 3 was purchased from Pharmingen.

Plasmids

Plasmids of GST–c-Jun(1–79), HA–JNK1, MEKK1, Flag–HPK1, Flag–HPK1-M(46), were described previously (Hu et al., 1996; Meyer et al., 1996). Flag–HPK1(D385N) was constructed by changing HPK1's aspartic acid residue 385 to an asparagine using polymerase chain reaction (PCR)-mediated site-directed mutagenesis, and inserted into the pCR3.1 expression plasmid (Invitrogen). Flag–HPK1-N(1-385) and Flag–HPK1-C(386-833) were generated by PCR and inserted into the pTOPO3.1 vector (Invitrogen). The bacteria-expressing constructs of fusion proteins GST–Crk and GST–Grb2 were kindly provided by SM Feller (Bavarian Julius-Maximilians University, Wuzburg, Germany) and by EY Skolnik (New York University Medical Center, New York, USA), respectively.

In vitro *caspase cleavage assays*

^{35}S-methionine-labeled Flag-HPK1 proteins (wild-type and the D385N mutant) were synthesized by an *in vitro* transcription and translation kit (Promega Biotech) as described previously (Ling et al., 1999). Labeled HPK1 proteins were immunoprecipitated from the reaction mixture with an anti-Flag antibody (M2) and protein G-agarose beads to remove endogenous proteases in reticulocyte lysate. The precipitated proteins were incubated in 50 μl of caspase reaction buffer (10 mM HEPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, pH 7.2) with recombinant caspase 3 (100 ng). The reaction was carried out at 37°C for indicated times, and terminated by adding SDS sample buffer and boiling for 5 min. The samples were resolved by SDS–PAGE (10%) and analysed by autoradiography.

Cell transfection and cell extracts preparation

The cells were plated 24 h before transfection at a density of 1.5×10^5 per 35-mm well. The 293 cells were transfected by a calcium phosphate precipitation protocol (Specialty Media), and HeLa cells were transfected using the Lipofectamine Plus reagent (Gibco) according to the manufacturer's instructions.

Whole cell lysate was prepared by suspending 2×10^6 cells in 150 μl lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM Na_3VO_4). The cell lysates were kept on ice and vigorously vortexed every 5 min for 20 min. The lysate was cleared by centrifugation at 15 000 *g* for 3 min, and stored at −80°C.

Immunocomplex kinase assays

The immunocomplex kinase assay for JNK activity was carried out as described (Chen et al., 1996b). For the HPK1

assays, endogenous HPK1 was precipitated with a specific antibody and protein A-agarose beads (Bio-Rad) in lysis buffer at 4°C for 2 h. The precipitates were washed twice with lysis buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-Cl [pH 7.6], and 0.1% Triton X-100), and twice with kinase buffer (20 mM 4-morpholinepropane-sulfonic acid [MOPS; pH 7.6], 2 mM EGTA, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mM dithiothrietol [DTT], and 1 mM Na_3VO_4), then mixed in 30 μl of kinase buffer containing 50 μM of ATP, and 10 μCi of [γ-^{32}P]ATP. Five μg of MBP was added per reaction as a substrate. The reaction was performed at 30°C for 30 min, then terminated by adding SDS sampling buffer. The reaction mixtures were boiled and analysed by SDS–PAGE and autoradiography.

In vitro *interaction assays*

Five hundred μg of cellular proteins from untreated or anti-Fas-treated Jurkat cells was incubated with 20 μg of purified GST-Crk (or GST-Grb2) proteins and 25 μl packed GSH-conjugated agarose beads in 1.5 ml of the lysis buffer at 4°C for 2 h with continuous rotation. The affinity-purified complex were then washed three times with 1.5 ml of the lysis buffer, denatured by adding 70 μl of 1 × SDS sampling buffer and 5-min boiling, and analysed by Western blot assays using anti-HPK1 antibodies.

Western blot analyses

For Western blot analyses, the samples were prepared as described above. The samples were resolved by SDS–PAGE, and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with a primary antibody (anti-HPK1 [Ab2025, Ab484, or Ab1404], 1:1000 dilution), washed, and blotted with a secondary antibody conjugated with HRP (1:1000 dilution). The membrane was then developed in the ECL reagent (Amersham) and exposed to an X-ray film.

Abbreviations

Crk, CT10 regulator of kinase; Grb2, growth factor receptor bound protein 2; GST, glutathione S-transferase; HPK1, hematopoietic progenitor kinase 1; JNK, c-Jun N-terminal kinase; kD, kilodalton; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEKK1, MAPK kinase kinase 1; MKK, MAPK kinase; PAK, p21-GTPase-activated kinases; z-DEVD-FK, z-Asp-Glu-Val-Asp-fluoromethyl ketone; z-VAD-FK, z-Val-Ala-Asp-FK.

Acknowledgments

We thank Drs SM Feller, MC-T Hu, and EY Skolnik for generous gifts, members of Tan laboratory for their helpful discussions and critical reading of this manuscript, S Lee and R Afshar for technical assistance, and M Lowe for secretarial assistance. This work was supported by the National Institutes of Health grants R01-AI38649 and R01-AI42532 (to T-H Tan). T-H Tan is a Scholar of the Leukemia Society of America. Y-R Chen was supported by a Department of Defense Predoctoral Fellowship (DAMD17-97-1-7078) in the Breast Cancer Research Program, and is a recipient of Department of Defense Postdoctoral Fellowship (DAMD17-99-1-9507) in the Prostate Cancer Research Program. CF Meyer was supported by an NIH postdoctoral Trainingship in Immunology.

References

Anafi M, Kiefer F, Gish GD, Mbamalu G, Iscove NN and Pawson T. (1997). *J. Biol. Chem.*, **272,** 27804–27811.

Bagrodia S, Derijard B, Davis RJ and Ceroine RA. (1995). *J. Biol. Chem.*, **270,** 27995–27998.

Brenner B, Koppenhoefer U, Weinstock C, Linderkamp O, Lang F and Gulbins E. (1997). *J. Biol. Chem.*, **272,** 22173–22181.

Cardone M, Salvesen GS, Widmann C, Johnson G and Frisch SM. (1997). *Cell,* **90,** 315–323.

Chen Y-R, Meyer CF and Tan T-H. (1996a). *J. Biol. Chem.*, **271,** 631–634.

Chen Y-R, Wang W, Kong A-NT and Tan T-H. (1998). *J. Biol. Chem.*, **273,** 1769–1775.

Chen Y-R, Wang X, Templeton D, Davis RJ and Tan T-H. (1996b). *J. Biol. Chem.*, **271,** 31929–31936.

Deak JC, Cross JV, Lewis M, Qian Y, Parrott LA, Distelhorst CW and Templeton DJ. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 5595–5600.

Diener K, Wang XS, Chen C, Meyer CF, Bray J, Zukowski M, Tan T-H and Yao Z. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 9687–9692.

Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbabda S, Robertson M, Ghayur T, Wong WW, Kamen R, Weichselbaum R and Kufe D. (1995). *EMBO J.,* **14,** 6148–6156.

Erhardt P, Tomaselli KJ and Cooper GM. (1997). *J. Biol. Chem.*, **272,** 15049–15052.

Fanger GR, Gerwins P, Widmann C, Jarpe MB and Johnson GL. (1997). *Curr. Opin. Genet. Dev.*, **7,** 67–74.

Goillot E, Raingeaud J, Ranger A, Tepper RI, Davis RJ, Harlow E and Sanchez I. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 3302–3307.

Graves JD, Gotoh Y, Draves K, Ambrose D, Han DKM, Wright M, Chernoff J, Clark EA and Krebs EG. (1998). *EMBO J.*, **17,** 2224–2234.

Hu MC-T, Qiu WR, Wang X, Meyer CF and Tan T-H. (1996). *Genes & Dev.,* **10,** 2251–2264.

Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K and Gotoh Y. (1997). *Science,* **275,** 90–94.

Ip YT and Davis RJ. (1998). *Curr. Opin. Cell Biol.*, **10,** 205–219.

Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A and Green DR. (1998). *Mol. Cell,* **1,** 543–551.

Kiefer F, Tibbles LA, Anafi M, Janssen A, Zanke BW, Lassam N, Pawson T, Woodgett JR and Iscove NN. (1996). *EMBO J.*, **15,** 7013–7025.

Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ and Williams LT. (1997). *Science,* **278,** 294–298.

Kyriakis JM and Avruch J. (1996). *J. Biol. Chem.*, **271,** 24313–24316.

Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG and Earnshaw WC. (1994). *Nature,* **371,** 346–347.

Lazebnik YA, Takahashi RD, Moir RD, Goldman RD, Poirier GG, Kaufmann SH and Earnshaw WC. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 9042–9046.

Lenczowski JM, Lourdes D, Eder AM, King LB, Zacharchuk CM and Ashwell JD. (1997). *Mol. Cell. Biol.*, **17,** 170–181.

Levkau B, Koyama H, Raines EW, Clurman BE, Herren B, Orth K and Roberts JM. (1998). *Mol. Cell,* **1,** 553–563.

Ling P, Yao Z, Meyer CF, Wang XS, Oehrl W, Feller SM and Tan T-H. (1999). *Mol. Cell. Biol.*, **19,** 1359–1368.
Meyer CF, Wang X, Chang C and Tan T-H. (1996). *J. Biol. Chem.*, **271,** 8971–8976.
Oehrl W, Kardinal C, Ruf S, Adermann K, Groffen J, Feng G-S, Blenis J, Tan T-H and Feller SM. (1998). *Oncogene,* **17,** 1893–1902.
Park DS, Stefanis L, Irene Yan CY, Farinelli SE and Greene LA. (1996). *J. Biol. Chem.*, **271,** 21898–21905.
Pombo CM, Kehrl JH, Irma S, Woodgett JR, Force T and Kyriakis JM. (1995). *Nature,* **377,** 750–754.
Rehemtulla A, Hamilton CA, Chinnaiyan AM and Dixit VM. (1997). *J. Biol. Chem.*, **272,** 25783–25786.
Rudel T and Bokoch GM. (1997). *Science,* **276,** 1571–1574.
Salvesen G and Dixit VM. (1997). *Cell,* **91,** 443–446.
Seimiya H, Mashima T, Toha M and Tsuruo T. (1997). *J. Biol. Chem.*, **272,** 4631–4636.
Shiah S-G, Chuang S-E, Chau Y-P, Shen S-C and Kuo M-L. (1998). *Cancer Res.*, **59,** 391–398.
Song O, Lees-Miller SP, Kumar S, Zhang N, Chan DW, Smith GCM, Jackson SP, Alnemri ES, Litwack G, Khanna KK and Lavin MF. (1996). *EMBO J.*, **15,** 3238–3246.
Steller H. (1995). *Science,* **267,** 1445–1449.
Thompson CB. (1995). *Science,* **267,** 1456–1462.
Tournier C, Whitmarsh AJ, Vavanagh J, Barret T and Davis RJ. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 7337–7342.
Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z and Kolesnick RN. (1996). *Nature,* **380,** 75–79.
Wang W, Zhou G, Hu MC-T, Yao Z and Tan T-H. (1997). *J. Biol. Chem.*, **272,** 22771–22775.
Wen L-P, Fahrni JA, Troie S, Guan J-L, Orth K and Rosen GD. (1997). *J. Biol. Chem.*, **272,** 26056–26061.
Widmann C, Gerwins P, Johson NL, Jarpe MB and Johnson GL. (1998). *Mol. Cell. Biol.*, **18,** 2416–2429.
Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenberg ME. (1995). *Science,* **270,** 1326–1331.
Yao Z, Brown A, Wang XS, Zhou G, Diener K, Zukowski M and Tan T-H. (1997a). *J. Biol. Chem.*, **272,** 2118–2125.
Yao Z, Diener K, Wang XS, Zukowski M, Matsumoto G, Zhou G, Tibbles LA, Sasaki T, Nishina H, Tan T-H, Woodgett J and Penninger JM. (1997b). *J. Biol. Chem.*, **272,** 32378–32383.
Zanke BW, Boudreau K, Rubie E, Winnett E, Tibbles LA, Zon L, Kyriakis J, Liu F-F and Woodgett JR. (1996). *Curr. Biol.*, **6,** 606–613.
Zhou G, Lee SC, Yao Z and Tan T-H. (1999). *J. Biol. Chem.*, **274,** 13133–13138.

INTERNATIONAL JOURNAL OF ONCOLOGY 16: 651-662, 2000

The c-Jun N-terminal kinase pathway and apoptotic signaling (Review)

YI-RONG CHEN and TSE-HUA TAN

Department of Immunology, Baylor College of Medicine, Houston, TX 77030, USA

Received December 20, 1999; Accepted January 28, 2000

Abstract. The c-Jun N-terminal kinase (JNK) group of mitogen-activated protein kinases (MAPKs) is activated in mammalian cells by environmental stress, pro-inflammatory cytokines, and mitogenic stimuli. Biochemical and genetic studies demonstrate that JNK regulates the activities of many transcription factors, and that the JNK pathway is required for the regulation of inflammatory responses, cell proliferation, and apoptosis. The involvement of JNK in apoptotic cell death is particularly intriguing, and has been actively studied in recent years. An improved understanding of JNK-mediated apoptotic signaling may provide novel strategies in prevention and treatment of cancers.

Contents

1. Introduction

Mitogen-activated protein kinases (MAPKs) are conserved signaling molecules that exist in various organisms, from yeasts to mammals (1). Mammalian MAPKs consist of three major groups including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs, also known as stress-activated protein kinases, SAPKs), and p38-MAPKs (1). MAPKs share a common character: they are activated by phosphorylation at a Thr-X-Tyr motif (X is Glu in ERKs, Pro in JNKs, and Gly in p38-MAPKs) in kinase subdomain VIII (1). The major targets for MAPK kinases are transcription factors that regulate gene expression. Recently, rapid progress has been made in the identification and cloning of regulators of the JNK pathway. The known JNK pathway consists of JNKs and various MAP2Ks, MAP3Ks, and MAP4Ks, which may mediate signals induced by distinct stimuli (Fig. 1). Activated JNK can be dephosphorylated and inactivated by a number of dual-specificity phosphatases. The JNK pathway responds to many diverse stimuli (1,2) including mitogens, fluid shearing, pro-inflammatory cytokines, and environmental stresses including various apoptotic stimuli. These diverse cellular functions may be achieved with the subtle regulation of the JNK pathway by its regulators in conjunction with other signaling pathways. In this review, we summarize recent studies on the JNK pathway and the involvement of the JNK pathway in apoptotic signaling.

Correspondence to: Dr Tse-Hua Tan, Department of Immunology, M929, One Baylor Plaza, Houston, TX 77030, USA

Key words: mitogen-activated protein kinase, c-Jun N-terminal kinase, apoptosis, cell signaling

2. The JNK pathway

JNKs. The human JNKs are encoded by three genes *jnk1*, *jnk2*, and *jnk3* (3-6). The corresponding murine genes have also been identified (7). JNK1 and JNK2 are widely expressed in many tissues, while JNK3 is preferentially expressed in neuronal tissues. Ten isoforms of JNK are generated by alternative splicing of the transcripts from the three genes (6). It has been suggested that JNK isoforms may target different substrates *in vivo*. Most of the known substrates for JNK are transcriptional factors including c-Jun, JunD, ATF-2, ATFa, Elk-1, and Sap-1a (2). Generally, phosphorylation of these factors by JNK increases their transcriptional activity.

The physiological functions of JNK have been examined by genetic analysis. The *jnk1*$^{-/-}$, *jnk2*$^{-/-}$, and *jnk3*$^{-/-}$ single mutant mice have no global abnormality (8-10). The T cells in *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ mice preferentially differentiate into Th2 rather than Th1 cells (9,10). The *jnk1*$^{-/-}$ T cells hyper-proliferate and exhibit decreased activation-induced apoptosis (10). Excitotoxicity-induced apoptosis in the hippocampus is absent in *jnk3*$^{-/-}$ mice in comparison to normal mice (8). The *jnk1/jnk3* and *jnk2/jnk3* deficient mice also develop normally (11); however, *jnk1/jnk2* deficient mice are embryonically lethal and have severe dysregulation of apoptosis in the brain (11). These results indicate that JNK1 and JNK2 may have overlapping functions, and are important in regulation of immune response and embryonic development. JNK3 may have its unique functions in the neuronal tissues.

MAP2Ks in the JNK pathway. JNK is activated by phosphorylation at amino acids Thr-183 and Tyr-185. MKK4 (also known as SEK1 or JNKK1) phosphorylates and activates

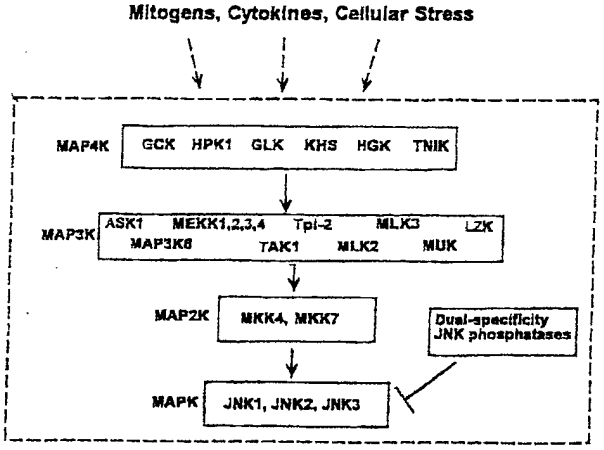


Figure 1. The JNK signaling pathway. Currently known MAPKs, MAP2Ks, MAP3Ks, and MAP4Ks of the JNK pathway are illustrated schematically. Activated JNK can be dephosphorylated and down-regulated by dual-specificity phosphatases. The signaling specificity among the components is not presented in this figure.

JNK *in vitro* and *in vivo* (12-14). However, recombinant MKK4 does not show apparent dual-specificity. Recombinant wild-type JNK proteins are phosphorylated at Tyr, Ser and Thr residues in the presence of recombinant MKK4, whereas a kinase-inactive JNK is phosphorylated predominantly on Tyr (12). The phosphorylation on Thr-183 may be caused by the proline-directed kinase activity of JNK itself, occurring after MKK4-mediated Tyr-phosphorylation. It is also possible that MKK4 obtains dual-specific kinase activity after activation by upstream kinases. Homologous deletion in *mkk4* genes is embryonically lethal in mice, indicating that MKK4 is essential for embryonic development (15,16). MKK4 is mutated in several tumors, suggesting that MKK4 is a potential tumor suppressor (17,18).

Recently, a novel kinase, MKK7 (also named as JNKK2), has been cloned and found to specifically activate JNK, but not p38-MAPK or ERK (19-22). MKK7 is related to MKK4 and belongs to the mammalian MAPK kinase superfamily (19,20). Unlike MKK4, MKK7 predominantly phosphorylates JNK at the threonine residue. It has been shown that these two MKKs activate JNK synergistically *in vitro*. However, it is unclear whether these two kinases cooperate in JNK activation *in vivo*. Both MKK4 and MKK7 are widely expressed in human and murine tissue (19,20), and mediate signals from the same panel of extracellular stimuli (22).

MAP3Ks in the JNK pathway. Multiple MAP kinase kinase kinases (MAPKKKs or MAP3Ks) have been reported to activate the JNK pathway via MKK4 and/or MKK7. These kinases include MEKK1-4, ASK1/MAPKKK5, MAPKKK6, TAK1, Tpl-2/Cot, and mixed lineage kinases. The four MEKKs (ranging from 69.5-195 kDa in size) have homologous kinase domains in the C-termini of the proteins, but have little homology in their N-termini. All four MEKKs (MEKK1-4) activate the JNK pathway (reviewed in ref. 23). MEKK1, MEKK2, and MEKK3 also activate the ERK pathway (24-26), and activate the NF-κB through the IκB kinases (IKKs) (27,28). MEKK3 and MEKK4 have been shown to activate the p38-MAPK pathway through MKK6 (29,30).

TGF-ß activated kinase 1 (TAK1) is activated by TGF-ß (31), interleukin 1 (32), ceramide, and UV-C treatments (33). TAK1 activates JNK and p38-MAPK, but has no effect on ERK (31,34). TAK1 also activates IKK and NF-κB transcriptional activity (32). TAK1 and its associated protein, TAB1, are important in the dorsoventral patterning of early *Xenopus* embryos (35).

Apoptosis signal-regulating kinase 1 (ASK1, also named MAPKKK5) has been shown to activate JNK and p38-MAPK through MKK4 and MKK3, respectively (36,37). An ASK1-related kinase kinase, MAPKKK6 (murine homologue is ASK2), was identified by yeast two-hybrid screen using ASK1 as a bait (38). MAPKKK6 also interacts with ASK1 when coexpressed in 293 cells (38). In contrast to ASK1, MAPKKK6 only weakly activates JNK1 but does not activate p38-MAPK or ERK (38).

Murine tumor progression locus 2 (Tpl-2), a homolog of the human transforming gene *cot*, is a proto-oncogene involved in T lymphomas induced by Moloney murine leukemia virus (39). Expression of Tpl-2 in mammalian cells activates ERK and JNK through the direct phosphorylation of MEK-1 and MKK4, respectively (40).

The mixed lineage kinase (MLK) family of kinases includes MLK1 (41), MLK2/mammalian STE20-like (MST) (41,42), MLK3/src-homology 3 (SH3) domain-containing proline-rich kinase (SPRK)/protein tyrosine kinase 1 (PTK-1) (43-45), MAPK-upstream kinase (MUK)/dual leucine zipper-bearing kinase (DLK)/leucine-zipper protein kinase (ZPK) (46,47), and leucine zipper-bearing kinase (LZK) (48). This group of kinases contains similar catalytic domains, which show structural features of both tyrosine- and serine/threonine-specific protein kinases, an SH3 motif at the N-terminus and proline-rich regions at the C-terminus. MLKs also have Leu/Ile-zipper motifs near the C-terminus. These motifs may allow MLKs to dimerize or interact with other molecules. MLK2 and MLK3 have been shown to interact with Cdc42 and Rac proteins (45,49). MLK2/MST, MLK3, MUK and LZK activate the JNK pathway when co-expressed with JNK in mammalian cells (50). MLK2/MST also weakly activates p38-MAPK and ERK (51). MLK3 activates the p38-MAPK pathway via MKK3 and MKK6, but has no effect on the ERK pathway (44).

JNK-activating STE20-related kinases. The MAP kinase modules in *S. cerevisiae* are controlled by a MAP4K named STE20. Several kinases containing an STE20-like kinase domain have been identified in mammalian cells, and can be divided into several subgroups, which roughly correspond to their structures and biochemical properties. Among them, GCK, HPK1, KHS/GCKR, GLK, HGK/NIK, have been shown to regulate the JNK pathway through MAP3Ks. Therefore, they can be classified as MAP4Ks in mammalian cells.

Germinal center kinase (GCK), hematopoietic progenitor kinase 1 (HPK1), kinase homologous to STE20 (KHS)/GCK related kinase (GCKR), and GCK-like kinase (GLK) form a subgroup of STE20-like kinases, which share a similar structure. They have the STE20-like catalytic domain in their N-terminus and at least 2 proline-rich motifs (Src homology 3

[SH3] domain binding sites) in their middle region. A domain distantly related to part of murine citron protein (citron homology domain, CNH domain) is located in the C-terminus of these kinases. GCK was first found to be expressed in B lymphocytes residing in the germinal center region of lymphoid follicles (52), but was later found to be ubiquitously expressed in many tissues. GCK has been shown to interact with TNF receptor-associated factor-2 (TRAF2) and with MEKK1 (53). Therefore, GCK may link the TNF receptor complex to the JNK pathway through MEKK1. GCK also interacts with the small G protein Rab8 (54); however, the biological significance of this interaction is unclear.

HPK1 is preferentially expressed in hematopoietic cells, especially in lymphocytes (55,56). HPK1 contains four proline-rich motifs, and interacts with the adaptor molecules Grb2, Crk, CrkL, Nck, and HIP55/SH3P7 (57-60). These adaptor proteins bind to tyrosine-phosphorylated proteins through their SH2 domains. The interaction between HPK1 and adaptor proteins may recruit HPK1 to surface receptor-tyrosine kinase complexes. Indeed, HPK1 is activated by tyrosine phosphatase inhibitors and is tyrosine-phosphorylated after epidermal growth factor stimulation and T-cell receptor ligation (57,58). HPK1 interacts with MEKK1 *in vivo* and directly phosphorylates its regulatory region *in vitro* (55). HPK1 also interacts with MLK3 and TAK1 (56,61). HPK1 is upstream of TAK1 in TGF-ß-induced JNK activation (61).

KHS/GCKR is activated by TNF-α and UV irradiation (62,63). A KHS/GCKR dominant-negative mutant or antisense suppresses TNF-α, TRAF2, and UV-induced JNK activation (63). KHS/GCKR also physically interacts with TRAF2 (63). Through its proline-rich domains, KHS/GCKR interacts with the SH3 domains of Crk and CrkL, but not with the SH3 domains of Grb2 or Nck (59). KHS/GCKR is constitutively active in chronic myeloid leukemia (CML) cells and interacts with oncoprotein Bcr-Abl (64). KHS/GCKR is activated by Bcr-Abl in a Ras-dependent manner (64). A dominant-negative KHS/GCKR blocks Bcr-Abl-induced JNK activation (64).

GLK is widely expressed in many tissues (65). GLK-induced JNK activation is blocked by a dominant-negative mutant of MKK4 or MEKK1 (65). GLK phosphorylates recombinant MEKK1 (65). These data suggest that GLK regulates the JNK pathway through MEKK1 and MKK4. To date, GLK is known to be regulated by UV irradiation and TNF-α (65). Since GLK also contains proline-rich motifs, like other kinases in this family, it may be regulated through interaction with SH3 domain-containing molecules.

HPK1/GCK-like kinase (HGK)/Nck-interacting kinase (NIK) and closely related Traf-2 and Nck-interacting kinase (TNIK) also contain citron-homology (CNH) domains; however, their sequences share low homology to the CNH domains in HPK1, GCK, KHS, and GLK. HGK/NIK and TNIK also contain a coiled-coil domain that is not found in kinases of the GCK/HPK1 subgroup. HGK and its murine counterpart, NIK, are 98% identical except for an insertion containing two proline-rich motifs in the middle region of NIK (66,67). A longer form of human HGK that contains proline-rich motifs was also detected in brain tissue by RT-PCR (66). However, the short form of HGK appears to be the predominant form in other human tissues including liver, skeletal muscle and placenta (66). Murine HGK/NIK activates JNK and MKK4 when co-expressed in cells, and interacts with MEKK1 (67). Murine HGK/NIK strongly interacts with the SH3 domain of Nck, but not with other SH3 domain-containing molecules, such as Grb2 and phospholipase C-γ (67). Human HGK/NIK-induced JNK activation can be blocked by a dominant-negative mutant of MKK4, MKK7, or TAK1, but not by a dominant-negative MEKK1 mutant (66). The difference between human and murine HGK/NIK (long versus short forms) is intriguing and needs to be further examined.

TNIK, as the name indicates, interacts with TRAF2 and Nck. TNIK activates JNK but not p38-MAPK or ERK. Over-expression of TNIK results in the disruption of F-actin and the inhibition of cell spreading. TNIK also phosphorylates gesolin *in vitro* (68). These data suggest that TNIK may be involved in the regulation of cytoskeleton signaling; however, whether this function is dependent on the JNK-activating ability of TNIK is unclear.

Scaffold proteins. The MAPK kinase pathways in *S. cerevisiae* are coordinated by scaffold proteins such as Ste5p protein and Pbs2p, (69,70). Recent studies show that mammalian cells also contain proteins that serve as scaffolds. JNK-interacting protein 1 (JIP1) was isolated by a two-hybrid screen for proteins that bind to JNK. JIP2 was isolated from human brain cDNA library using JIP1 cDNA as a probe (71). JIPs bind to JNK, but not to ERK or p38-MAPK (71,72). Both JIP1 and JIP2 bind to MKK7 but not MKK4 (71). JIP1 also selectively interacts with the MLK family of MAP3Ks. MKK7 and MLK bind to regions on JIP1 that are distinct from the JNK binding site (71,72). HPK1 also interacts with JIP1; however, whether this interaction is direct or mediated through MLK family members is uncertain (72). JIP1 and JIP2 interact to form oligomeric complexes that locate in peripheral cytoplasmic projections extended at the cell surface (71). These results suggest that JIPs may facilitate the aggregation of components in the JNK pathway and enhance signal transmission.

JNK/SAPK-associated protein 1 (JSAP1) was isolated by a yeast two-hybrid screen using JNK3 as a bait (73). JSAP1 interacts with JNKs but not with p38-MAPKs or ERKs. JSAP1 is predominantly expressed in brain tissues. Among JNK isoforms, JSAP1 shows higher affinity toward JNK3 (73), which is also predominantly expressed in brain. JSAP1 interacts with MKK4 and MEKK1. The regions of JSAP1 that bind JNK, MKK4, and MEKK1 are distinct from one another. Furthermore, co-expression of JSAP1 enhances JNK activation by the MEKK1-MKK4 pathway (73). These results suggest that JSAP1 may be a scaffold protein for JNK3 activation in brain tissues. Some kinases in the JNK pathway have extended regulatory domains, which may enable them to serve as scaffold proteins. For example, MEKK1 is capable of interacting with JNK (74), MKK4 (67), NIK (murine HGK) (67), and HPK1 (55) through its different regions. These properties enable MEKK1 to serve as a kinase and a scaffold protein in the JNK pathway.

Dual-specificity phosphatases. MAP kinases are regulated by their own phosphorylation status. The phosphorylation on the T-P-Y motif can be removed by dual-specificity phosphatases, resulting in the inactivation of MAP kinases (reviewed in refs. 75,76). Several phosphatases including PAC-1, MKP-1,

MKP-2, MKP-4, MKP-5, and M3/6 suppress JNK activation in transfection studies (77-81). It has been shown that the expression and activity of these dual-specificity phosphatases are increased after mitogenic signaling (82-85), and that both ERK and JNK can induce the expression of some dual-specificity phosphatases (85-87). The induction of expression and activity of these phosphatases by mitogenic signals and MAPKs may, in turn, lead to down regulation of MAPK activities (including JNKs and ERKs). This could be a negative feedback loop to ensure a transient MAPK activation by mitogenic signals.

3. JNK in apoptotic signaling

Involvement of JNK in apoptotic signaling. Apoptosis is important in regulating development and maintaining homeostasis in multicellular organisms (reviewed in refs. 88,89). Cells undergo apoptosis in response to ligation of surface death receptors (e.g., TNF-α receptor, Fas), depletion of growth and survival factors, DNA-damaging agents, and many other stimuli (89). Apoptosis is regulated by genetic and biochemical programs. Caspases (aspartate-directed cysteine proteases) are required for apoptosis (90). Caspases cleave many cellular proteins and play major roles in causing the morphological and biochemical changes seen in apoptotic cells. The blocking of caspase activation by peptide inhibitors or by viral proteins, suppresses apoptosis progression (reviewed in ref. 90). To date, the known signaling pathways that mediate activation of caspase cascades are initiated either at death receptors or at the mitochondria. The mechanisms of caspase activation in response to most apoptotic stimuli, especially extreme stress, are being actively studied.

JNK activation is observed in apoptosis induced by a variety of stimuli in different cell types. JNK is activated by growth factor withdrawal, ligation of death receptors, heat shock, ceramides, UV radiation, γ radiation, dopamine, paclitaxel, retinoids, ischemia-reperfusion, intracellular acidification, pro-oxidants, and chemopreventive and therapeutic agents for cancer (summarized in Table I). JNK is also activated in apoptosis induced by forced expression of Bruton's tyrosine kinase (91), Myc (92), polyglutamine-expanded huntingtin (93), and BRCA1 (94). Forced expression of JNK or upstream activators of JNK in mammalian cells leads to apoptosis (95,96). Interference with the JNK pathway by antisense oligos to JNK or the dominant-negative mutant of MEKK1, MKK4/SEK, or JNK1 suppresses apoptosis (95-98). JNK's substrate, c-Jun, is required for ceramide-induced apoptosis (99) and apoptosis of neuronal cells caused by NGF withdrawal (100,101). These results indicate the importance of the JNK pathway in apoptotic signaling.

Several genetic studies in animals also support the role of JNK in apoptotic signaling. JNK3-deficient mice are resistant to excitotoxicity-induced neuron apoptosis (8). In addition, the *jnk1*$^{-/-}$ T cells hyper-proliferate and exhibit decreased activation-induced apoptosis (10). The *jnk1/jnk3* and *jnk2/jnk3* deficient mice develop normally (11); however, *jnk1/jnk2* deficient mice are embryonically lethal and have severe dysregulation of apoptosis in the brain (11). Mice whose endogenous c-jun has been replaced by a mutant jun alelle with serines 63 and 73 (the major JNK targets) mutated to alanines (*Jun AA*) have been generated (102). *Jun AA* mice are viable and fertile, but resistant to neuronal apoptosis induced by excitatory amino acid kainate. *Jun AA* fibroblasts are also resistant to UV-induced apoptosis (102).

Table I. JNK activation by apoptotic stimuli.

Growth factors withdrawal
NGF (**95**[a],132,153)
Erythropoietin (**119**)
Ligation of death receptors
Fas (105,**106-108**), TNF-α (103), TRAIL (167,168)
Radiation
UV radiation (3,**96**,**98**), γ radiation (**96**,109,130,169)
Chemopreventive and therapeutic agents for cancer
ara-C (131,170), paclitaxel (115,171,180), retinoids (120,**172**), vitamin E succinate (112), cisplatin (98,114), etoposide (**151**), isothiocyanates (113,181), adriamycin (182)
Miscellaneous
HIV1 gp120 (173), heat shock (7,**98**,**124**), ceramides (**97**,**107**,**174**), proteasome inhibitors (116), dopamine (117), ligation of MHC class I (**175**), ischemia-reperfusion (176,177), intracellular acidification (178), hydrogen peroxide (134,179)

[a]References reporting suppression of apoptosis by interfering with the JNK pathway are shown in bold.

Despite all the evidence for the involvement of JNK in apoptosis, the role of JNK in death receptor-mediated apoptosis is unclear. It has been reported that JNK activation by TNF-α is not involved in apoptosis induction (103,104), because JNK activation and apoptosis can be dissociated using different components in TNF-α receptor complex. The role of Fas-mediated JNK activation in apoptosis is also controversial (105-108). In different systems, JNK activation by Fas ligation has been shown to be either caspase-dependent or caspase-independent (96,105,106).

Duration of JNK activation may determine cell fate. The JNK pathway participates in cellular responses to mitogens, stresses, and apoptotic agents. How does the JNK pathway integrate cellular signaling to achieve these diverse functions? We have found that JNK in response to mitogenic and apoptotic signals exhibits different activation patterns, transient versus persistent, respectively (96,109). Co-treatment of T cells with a tyrosine phosphatase inhibitor (sodium orthovanadate) plus activation signals (PMA plus ionomycin) prolongs the JNK induction by T-cell activation agents and results in T cell apoptosis (96). This result shows the association between sustained JNK activation and apoptosis, and suggests that phosphatase activity is important in ensuring the transient JNK activation by mitogens. Because of the kinetic difference of JNK activation

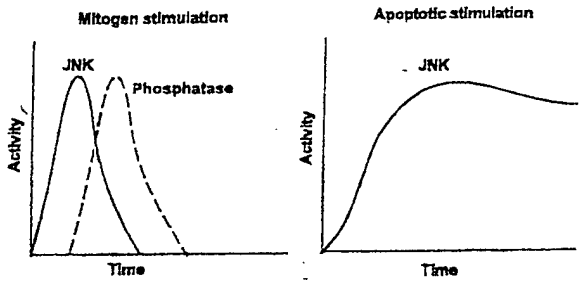


Figure 2. Duration of JNK activation may determine cell fate. Transient JNK activation leads to cell proliferation, whereas sustained JNK activation causes apoptosis. Dual-specific JNK phosphatases induced by mitogenic stimuli down-regulate JNK activity to ensure a transient JNK activation. In apoptotic cells, JNK phosphatases are either not expressed or suppressed. Therefore, JNK activation is not downregulated and cells undergo apoptosis.

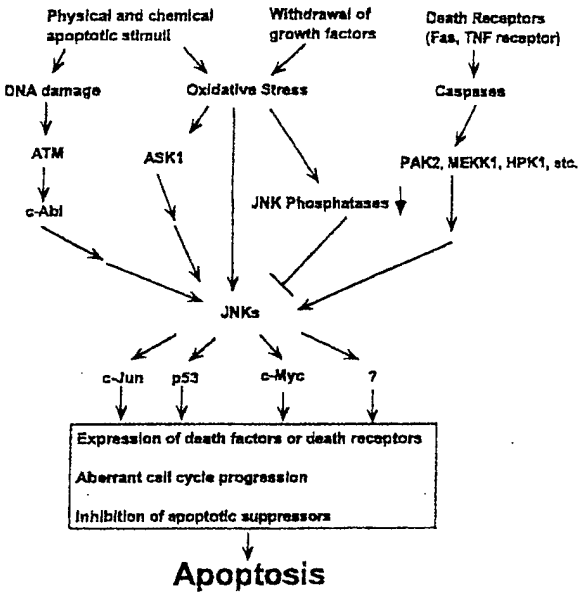


Figure 3. The mechanisms of JNK activation by apoptotic stimuli, and the downstream events of JNK-mediated apoptosis.

by mitogenic and apoptotic signaling, we proposed that the duration of JNK activation may determine cell fate (96). Transient JNK activation induces proliferation, but does not induce apoptosis. In contrast, sustained JNK activation results in apoptosis (Fig. 2).

A similar observation was reported involving TNF-α-induced JNK activation and apoptosis. TNF-α treatment alone induces transient JNK activation in most cell types (4,110), and usually does not cause apoptosis induction by itself (103,110). It has been shown that prolonging TNF-α-induced JNK activation by co-incubation of cells with TNF-α plus cycloheximide, actinomycin D, or orthovanadate causes apoptosis (110). Inhibition of the expression of MKP-1, a dual-specificity phosphatase that inactivates JNK, also potentiates TNF-α-induced JNK activation and apoptosis (111). This evidence is consistent with the hypothesis that sustained JNK activation leads to apoptosis. To date, this hypothesis has not been conclusively proven, because an experimental system that specifically controls JNK activation in a fine-tuned manner has not been established. However, numerous experimental data are consistent with this hypothesis, since sustained JNK activation is tightly associated with apoptosis induction (e.g., 92,95,106,110,112-120).

It has been shown that JNK is constitutively activated in T cells transformed by HTLV-1 (121) or a parasite *Theileria parva* (122). JNK is also persistently activated in cells containing constitutively active EGF receptors (123). Whether those cells have higher basal apoptotic rates is not reported; however, these data do suggest that the cells can tolerate high basal levels of JNK activity and survive. These observations are not necessarily contradictory to the hypothesis that persistent JNK activation induces cell death. It is possible that other changes occur in the transformation process that can counteract the apoptotic effects of JNK. In that case, JNK activation may actually help the proliferation of transformed cells.

Based on a study of NGF-withdrawal-induced apoptosis, Xia *et al* proposed that ERK and JNK/p38 MAPK have opposing functions, and that the dynamic balance between ERK and JNK/p38 MAPK determines cell fate (95). This hypothesis is supported by several other studies in various systems (119,124,125). However, several pieces of evidence suggest that the balance between ERK and JNK (or p38 MAPK) may not be the major factor that determines cell fate in other experimental systems. CD40 ligation, which provides a protecting signal for B cells, induces transient JNK activation without affecting ERK activity (126,127). This suggests that a transient imbalance between JNK and ERK will not cause apoptosis. Furthermore, treatment of T cells with activation agents along with a phosphatase inhibitor non-preferentially prolongs both JNK and ERK activation (96), but this co-treatment causes apoptosis in T cells, indicating that apoptosis occurs in the presence of persistent JNK and ERK activation.

4. Mechanisms of JNK activation by apoptotic signals

JNK is activated by a variety of apoptotic stimuli. It is very likely that different stimuli cause JNK activation through distinct mechanisms. It is also possible that one agent can cause JNK activation though multiple mechanisms. The mechanisms by which apoptotic stimuli induce JNK activation have been gradually unfolded (Fig. 3).

DNA damage is a possible origin of cellular signaling leading to JNK activation by physical or chemical agents. Ataxia-Telangiectasia mutated (ATM), a PI3 kinase-related protein kinase, is activated by DNA-damaging agents. It has been shown that JNK activation is defective in $ATM^{-/-}$ cells, which do not contain functional ATM, in response to ionizing radiation, suggesting that ATM is required for JNK activation in response to DNA damage (128). ATM may activate the JNK pathway through c-Abl tyrosine kinase, since ATM physically interacts with c-Abl in response to DNA damage (129). In addition, c-Abl activates the JNK pathway, and JNK activation by DNA-damaging agents is defective in $c\text{-}Abl^{-/-}$ cells (130,131). Despite this evidence, whether ATM activation alone is sufficient to activate the JNK pathway in response to DNA damage is unclear.

It has been shown that oxidative stress mediates JNK activation in apoptosis induced by various stimuli (113,117, 132). It is unknown whether the reduction in antioxidants (GSH, thioredoxine) or the subsequent cellular damage caused by free radicals is the trigger of JNK activation and apoptosis. Recent reports indicate that several molecules in the JNK cascade are regulated by redox status directly or indirectly. JNK interacts with glutathion S-transferase p (GSTp), and this interaction suppresses JNK activity (133). Oxidation of GSTp causes the dissociation of JNK and GSTp and relieves the suppression of JNK activity (133). ASK1 is another candidate sensor of oxidative stresses (134,135). H_2O_2 induces dimerization or oligomerization of ASK1, and activates its kinase activity (135). Thioredoxine, a cellular antioxidant protein, binds to the N-terminus of ASK and inhibits ASK activity when in a reduced form (134). In contrast, oxidized thioredoxine fails to inhibit ASK activity. These results suggest that ASK1 may detect oxidative stresses through the redox status of associated thioredoxine. However, it is unknown whether thioredoxine-ASK1 mediates the triggering signal to the JNK pathway in most apoptotic conditions. It has not been shown that inhibition of ASK1 blocks JNK activation or apoptosis induced by oxidative stress. It will also be interesting to know whether purified thioredoxine-ASK1 alone can serve as a redox sensor.

JNK phosphatase activities are also important in controlling the duration of JNK activation. All known phospho-tyrosine phosphatases, including the dual-specificity phosphatases, contain an essential catalytic cysteinyl residue (76,136) and are, therefore, sensitive to thio (-SH)-reactive agents (137). Most apoptotic stimuli are known to generate oxidative stress (138) which, very possibly, will cause the oxidation of the sulfhydryl groups on these phosphatases and destroy their enzymatic activities. The expression of dual-specificity phosphatases is up-regulated by MAP kinase activities, which may explain the transient induction of MAPK by mitogenic stimulation. It is possible that apoptosis-mediated JNK activation does not induce the expression of these phosphatases, or that the oxidative environment during apoptosis can suppress the activity of those dual-specificity phosphatases, which in turn causes persistent JNK activation (Fig. 3).

The efficacy of interfering with the JNK pathway leading to the suppression of Fas-mediated apoptosis varies in different cell types. We and others found that JNK induced by Fas-ligation is caspase-dependent (96,105,139). These results indicate that JNK activation is a late event in Fas-mediated apoptosis, since caspases are considered effector molecules in apoptosis. However, many JNK upstream activators, including PAK2, MEKK1, and HPK1 are cleaved and activated by caspases (140-144), implying that this mechanism is conserved and, therefore, may be important for apoptosis. The cleavage and irreversible activation of JNK upstream activators by caspases may be a possible way to maintain a sustained JNK induction. However, we did detect persistent JNK activation by several apoptotic agents in the presence of a caspase inhibitor (113,120), suggesting that cleavage of upstream activators is not required for the maintenance of JNK activation. One possible explanation for our observation is that apoptotic-inducing agents were kept in the experimental system, therefore, providing a persistent stress to cells. In this situation, JNK may be persistently activated even without the cleavage of the upstream activators. On the other hand, cleavage of JNK activators may just serve as a mechanism for caspase-mediated JNK activation for some downstream apoptotic events, and may not play a role in maintenance of JNK activity.

Recently, a novel protein Daxx was identified by its interaction with the Fas death domain in a yeast two-hybrid screen. Daxx also interacts with the Fas death domain in a co-expression system in mammalian cells (145). Daxx induces JNK activation and apoptosis through physical interaction with ASK1 (146). This result suggests that Fas signaling can activate JNK independent of caspases. However, physical interactions between endogenous Daxx and Fas have not been reported in untransfected cell lines. In addition, Fas-mediated apoptosis is completely blocked in FADD- or caspase 8-deficient cells (147-149), suggesting that it is unlikely that any other signaling pathways mediate apoptotic signaling from Fas. Furthermore, deficiency in Daxx results in extensive apoptosis and embryonic lethality in mice (150). This result is strongly against a role for Daxx in promoting apoptosis.

5. Downstream targets of the JNK pathway in apoptosis

In most cases, JNK activation by apoptotic stimuli is independent of caspase activation (96,113,120,132), and it has been shown that the JNK pathway is required for caspase activation (116,151). Although some potential targets for JNK in apoptotic signaling have been identified (Fig. 3), the events downstream of JNK that lead to caspase activation are not completely clear.

Most of the known JNK substrates are transcription factors; therefore JNK may cause apoptosis through the induction of genes involved in apoptosis. The importance of N-terminal phosphorylation of the c-Jun protein in apoptosis induction is substantiated by a study which disrupted the JNK phosphorylation sites in endogenous c-Jun (102). Therefore, c-Jun may be a major mediator for JNK-induced apoptosis. It has been shown in various systems that JNK induction and the subsequent c-Jun activation enhance FasL expression (118, 152,153). Forced expression of a dominant-active MEKK1 causes up-regulation of FasL expression in Jurkat cells (118). DNA-damaging agents and withdrawal of survival factors also induce FasL expression and apoptosis via the activation of the JNK pathway (152,153). These results suggest that FasL expression is a major downstream event of JNK-mediated apoptotic signaling.

JNK also phosphorylates the tumor suppressor protein p53. Phosphorylation of p53 by JNK causes the dissociation of the MDM2-p53 complex, and prevents the ubiquitination and degradation of p53 (154). In addition, JNK signaling enhances p53-induced transcription and apoptosis (154). It is known that DNA-damaging agents enhance the levels of both p53 and surface Fas expression. p53 is also shown to facilitate surface Fas expression (155-157). However, we did not observe a correlation in JNK activation and p53-dependent Fas expression induced by various apoptotic stimuli (158). Fas expression can be induced in the absence of significant JNK activation, and JNK activation is not necessarily associated with Fas expression (158). Therefore, p53-dependent Fas expression may not be a downstream effect of JNK activation.

It is unclear whether JNK regulates the expression of other genes through the phosphorylation of p53.

Several pieces of evidence indicate that p53 and/or Fas-FasL interaction may not be essential for JNK-mediated apoptosis. First, activation of the JNK pathway induces apoptosis in p53-null cells, p53 mutant cells, and cells containing viral oncogenes that suppress p53 function (96,113, 120,158). These results suggest that functional p53 proteins may not be necessary for JNK-mediated apoptosis. Secondly, cells without FADD or containing dominant-negative FADD are resistant to Fas mediated apoptosis, but are still sensitive to DNA-damaging agents (147). This suggests that Fas/FasL, although induced, may not be required for apoptosis induced by DNA-damaging agents, which is JNK dependent. All of this evidence suggests that Fas-FasL interaction may not be essential for JNK-mediated apoptosis. However, whether JNK induces other death receptor signaling (e.g. DR4), which do not require FADD, needs to be further examined.

Recently, it has been shown that JNK interacts with and phosphorylates c-Myc proteins at residues Ser-62 and Ser-71 (159). Furthermore, a dominant-negative JNK1 impairs c-Myc-dependent apoptosis, and c-Myc (S62A/S71A)-expressing cells are more resistant to JNK-activating apoptotic stimuli including UV and paclitaxel than are c-Myc-expressing cells (159). These results suggest that c-Myc is a possible candidate that mediates JNK-induced apoptosis. Both c-Myc and c-Jun promote cell proliferation. The involvement of c-Myc and c-Jun in JNK-mediated apoptosis suggest that JNK may induce apoptosis by promoting untimely activation of some cell cycle regulators. Recently, JNK was found to be activated during the cell cycle, suggesting that JNK may participate in promoting cell cycle progression (160). The differential regulation of JNK induction in mitogenic and apoptotic responses strongly suggests that abnormal activation of mitogenic signaling may cause apoptosis, which is consistent with the hypothesis that apoptosis may be caused by an aberrant cell cycle progression (88,161).

JNK may induce apoptosis by inhibiting apoptotic suppressors. Phosphorylation of Bcl-2 has been suggested as a mechanism by which JNK induces apoptosis. The phosphorylation of Bcl-2 (Bcl-X_L) in the loop region suppresses Bcl-2 anti-apoptotic function (160,162,163). It has been shown that recombinant Bcl-2 is phosphorylated by immunoprecipited-JNK from cell extract. It has not been determined whether the phosphorylation of Bcl-2 is directly caused by JNK or a JNK-associated kinase (164). Co-expression of JNK and constitutively active Rac (a JNK activator) induces Bcl-2 phosphorylation (164). Blocking the JNK pathway suppresses Bcl-2 phosphorylation caused by paclitaxel treatment (160). However, Bcl-2 phosphorylation only occurs in apoptosis induced by certain agents such as microtubule disrupting chemicals (165,166). In addition, many apoptotic agents that induce JNK activation fail to cause Bcl-2 phosphorylation. These results suggest that JNK activation may result in Bcl-2 phosphorylation only under a specific condition.

6. Conclusion

Recently, major advances have been achieved in understanding the molecular mechanisms involved in the initiation and execution of apoptosis. The JNK pathway, a signaling mechanism that is involved in cellular responses to a variety of stimuli, also plays an important role in apoptotic signaling. The distinct patterns of JNK activation induced by apoptotic stimuli (persistent) and by other stimuli (transient) suggest that the duration of JNK activation determines the outcome of cellular signaling mediated by this pathway. The upstream signaling events that mediate JNK activation, which may be different in cells receiving distinct stimuli, are only partially revealed. The downstream molecules that link JNK to the execution of apoptotic cell death are also poorly understood. Further studies are required to understand how the JNK pathway interacts with the caspase cascade, and whether the apoptotic function of JNK is completely dependent on caspases, or if there is a caspase-independent pathway. The rapid progress in studying cellular signaling and apoptotic machinery may one day lead to the design of new preventative or therapeutic methods to treat illnesses caused by abnormalities in apoptotic cell death.

Acknowledgements

We thank the members of Tan laboratory for helpful discussion and critical reading of this manuscript and M. Lowe for secretarial assistance. T.-H. Tan is supported by NIH grants R01-AI42532 and R01-AI38649, as well as a Leukemia Society of America Scholar award. Y.-R. Chen is supported by a postdoctoral fellowship (DAMD17-99-1-9507) from the Department of Defense Prostate Cancer Research Program.

References

1. Schaeffer HJ and Weber MJ: Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol Cell Biol 19: 2435-2444, 1999.
2. Ip YT and Davis RJ: Signal transduction by the c-Jun N-terminal kinase (JNK)-from inflammation to development. Curr Opin Cell Biol 10: 205-219, 1998.
3. Derijard B, Hibi M, Wu I-H, Barrett T, Su B, Deng T, Karin M and Davis RJ: JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 76: 1025-1037, 1994.
4. Sluss HK, Barrett T, Derijard B and Davis RJ: Signal transduction by tumor necrosis factor mediated by JNK protein kinases. Mol Cell Biol 14: 8376-8384, 1994.
5. Kallunki T, Su B, Tsigelny I, Sluss HK, Derijard B, Moore G, Davis R and Karin M: JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. Genes Dev 8: 2996-3007, 1994.
6. Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard D and Davis RJ: Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J 15: 2760-2770, 1996.
7. Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J and Woodgett JR: The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369: 156-160, 1994.
8. Yang DD, Kuan C-Y, Whitmarsh A, Rincon M, Zheng TS, Davis RJ, Rakic P and Flavell RA: Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the *jnk3* gene. Nature 389: 865-870, 1997.
9. Yang DD, Conze D, Whitmarsh AJ, Barret T, Davis RJ, Rincon M and Flavell RA: Differentiation of $CD4^+$ T cells to Th1 cells requires MAP kinase JNK2. Immunity 9: 575-585, 1998.
10. Dong C, Yang DD, Wysk M, Whitmarsh AJ, Davis RJ and Flavell RA: Defective T cell differentiation in the absence of *jnk1*. Science 282: 2092-2095, 1998.
11. Kuan CY, Yang DD, Samanta RDR, Davis RJ and Flavell RA: The jnk1 and jnk2 protein kinases are required for regional specific apoptosis during brain development. Neuron 22: 667-676, 1999.

12. Sanchez I, Hughes RT, Mayer BJ, Yee K, Woodgett JR, Avruch J, Kyriakis JM and Zon LI: Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature 372: 794-798, 1994.
13. Derijard B, Raingeaud J, Barrett T, Wu I-H, Han J, Ulevitch RJ and Davis RJ: Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. Science 267: 682-685, 1995.
14. Lin A, Minden A, Martinetto H, Claret F-X, Lange-Carter C, Mercurio F, Johnson GL and Karin M: Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science 268: 286-290, 1995.
15. Nishina H, Fischer KD, Radvanyl L, Shahinian A, Hakem R, Rubie EA, Bernstein A, Mak TW, Woodgett JR and Penninger JM: Stress-signaling kinase SEK1 protects thymocytes from apoptosis mediated by CD95 and CD3. Nature 385: 350-353, 1997.
16. Yang D, Tournier C, Wysk M, Lu H-T, Davis RJ and Flavell RA: Targeted disrurtion of the MKK4 gene causes embryonic death, inhibition of c-Jun N-terminal kinase activation, and defects in AP-1 transcription. Proc Natl Acad Sci USA 94: 3004-3009, 1997.
17. Teng DH, Perry WL III, Hogan JK, Baumgard M, Bell R, Berry S, Davis T, Frank D, Frye C, Hattier T, Hu R, Jammulapati S, Janecki T, Leavitt A, Mitchell JT, Pero R, Sexton D, Schroeder M, Su PH, Swedlund B, Kyriakis JM, Avruch J, Bartel P, Wong AK, Tavtigian SV, *et al:* Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor. Cancer Res 57: 4177-4182, 1997.
18. Su GH, Hilgers W, Shekher MC, Tang DJ, Yeo CJ, Hruban RH and Kern SE: Alterations in pancreatic, biliary, and breast carcinomas support MKK4 as a genetically targeted tumor suppressor gene. Cancer Res 58: 2339-2342, 1998.
19. Tournier C, Whitmarsh AJ, Vavanagh J, Barret T and Davis RJ: Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH_2-terminal kinase. Proc Natl Acad Sci USA 94: 7337-7342, 1997.
20. Yao Z, Diener K, Wang XS, Zukowski M, Matsumoto G, Zhou G, Tibbles LA, Sasaki T, Nishina H, Tan T-H, Woodgett J and Penninger JM: Activation of stress-activated protein kinase/c-Jun N-terminal protein by a novel mitogen-activated protein kinase kinase (MKK7). J Biol Chem 272: 32378-32383, 1997.
21. Moriguchi T, Toyoshima F, Masuyama N, Hanafusa H, Gotoh Y and Nishida E: A novel SAPK/JNK kinase MKK7, stimulated by TNF-α and cellular stresses. EMBO J 16: 7045-7053, 1997.
22. Wu Z, Wu J, Jacinto E and Karin M: Molecular cloning and characterization of human JNKK2, a novel Jun NH_2-terminal kinase-specific kinase. Mol Cell Biol 17: 7407-7416, 1997.
23. Fanger GR, Gerwins P, Widmann C, Jarpe MB and Johnson GL: MEKKs, GCKs, MLKs, PAKs, TAKs, and Tpls: upstream regulator of the c-Jun amino-terminal kinases? Curr Opin Genet Dev 7: 67-74, 1997.
24. Lange-Carter CA, Pleiman CM, Gardener AM, Blumer KJ and Johnson GL: A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260: 315-319, 1993.
25. Blank JL, Gerwins P, Elliot EM, Sather S and Johnson GL: Molecular cloning of mitogen-activated protein/ERK kinase kinase (MEKK) 2 and 3. J Biol Chem 271: 5361-5368, 1996.
26. Ellinger-Ziegelbauer H, Brown K, Kelly K and Siebenlist U: Direct activation of the stress-activated protein kinase (SAPK) and extracellular signal-regulated protein kinase (ERK) pathways by an inducible mitogen-activated protein kinase/ERK kinase kinase 3 (MEKK) derivative. J Biol Chem 272: 2668-2674, 1997.
27. Lee FS, Hagler J, Chen ZJ and Maniatis T: Activation of the IκBα kinase complex by MEKK1, a kinase for the JNK pathway. Cell 88: 213-222, 1997.
28. Zhao Q and Lee F: Mitogen-activated protein kinase/ERK kinase kinase 2 and 3 activate nuclear factor-κB through IκB kinase-α and IκB kinase-ß. J Biol Chem 1999: 8355-8358, 1999.
29. Takekawa M, Posas F and Saito H: A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stress-induced activation of the p38 and JNK pathways. EMBO J 16: 4973-4982, 1997.
30. Deacon K and Blank JL: MEK kinase 3 directly activates MKK6 and MKK7, specific activators of the p38 and c-Jun NH2-terminal kinases. J Biol Chem 274: 16604-16610, 1999.
31. Yamaguchi K, Shirakabe K, Shibuya H, Kenji I, Oishi I, Ueno N, Taniguchi T, Nishida E and Matsumoto K: Identification of a member of the MAPKKK family as a potential mediator of TGF-ß signal transduction. Science 270: 2008-2011, 1995.
32. Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J-I, Cao Z and Matsumoto K: The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in the IL-1 signaling pathway. Nature 398: 252-256, 1999.
33. Shirakable K, Yamaguchi K, Shibuya H, Irie K, Matsuda S, Moriguchi T, Gotoh Y, Matsumoto K and Nishida E: TAK1 mediates the ceramide signaling to stress-activated protein kinase/ c-Jun N-terminal kinase. J Biol Chem 272: 8141-8144, 1997.
34. Wang W, Zhou G, Hu MC-T, Yao Z and Tan T-H: Activation of the hematopoietic progenitor kinase-1 (HPK-1)-dependent, stress-activated c-jun N-terminal kinase (JNK) pathway by transforming growth factor ß (TGF-ß)-activated kinase (TAK1), a kinase mediated of TGF-ß signal transduction. J Biol Chem 272: 22771-22775, 1997.
35. Shibuya H, Iwata H, Masuyama N, Gotoh Y, Yamaguchi K, Irie K, Matsumoto K, Nishida E and Ueno N: Role of TAK1 and TAB1 in BMP signaling in early *Xenopus* development. EMBO J 17: 1019-1028, 1998.
36. Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K and Gotoh Y: Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275: 90-94, 1997.
37. Wang XS, Diener K, Jannuzzi D, Trollinger D, Tan T-H, Lichenstein H, Zukowski M and Yao Z: Molecular cloning and characterization of a novel protein kinase with a catalytic domain homologous to mitogen-activated protein kinase kinase kinase. J Biol Chem 271: 31607-31611, 1996.
38. Wang XS, Diener K, Tan T-H and Yao Z: MAPKKK6, a novel mitogen-activated protein kinase kinase kinase, that associates with MAPKKK5. Biochem Biophys Res Commun 253: 33-37, 1998.
39. Patriotis C, Makris A, Bear SE and Tsichlis PN: Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. Proc Natl Acad Sci USA 90: 2251-2255, 1993.
40. Salmeron A, Ahmad TB, Calile GW, Pappin D, Narsimhan RP and Ley SC: Activation of MEK1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. EMBO J 15: 817-826, 1996.
41. Dorow DS, Devereux L, Dietzsch E and De Kretser T: Identification of a new family of human epithelial protein kinases containing two leucine/isoleucine-zipper domains. Eur J Biochem 15: 701-710, 1993.
42. Katoh M, Hirai M, Sugimura T and Terada M: Cloning and characterization of MST, a novel (putative) serine/threonine kinase with SH3 domain. Oncogene 10: 1447-1451, 1995.
43. Rena A, Gallo K, Godowski P, Hirai S, Ohno S, Zon L, Kyriakis JM and Avrich J: The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. J Biol Chem 271: 19025-19028, 1996.
44. Tibbles LA, Ing YL, Kiefer F, Chan J, Iscove N, Woodgett JR and Lassam NJ: MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. EMBO J 15: 7026-7035, 1996.
45. Teramoto H, Coso OA, Miyata H, Igishi T, Miki T and Gutkind JS: Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway: a role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. J Biol Chem 271: 27225-27228, 1996.
46. Hirai S-i, Izawa M, Osada S-I, Spyrou G and Ohno S: Activation of the JNK pathway by distantly related protein kinases, MEKK and MUK. Oncogene 12: 641-650, 1996.
47. Fan G, Merrit SE, Kortenjann M, Shaw PE and Holzman LB: Dual leucine zipper-bearing kinase (DLK) activates $p46^{SAPK}$ and $p38^{MAPK}$ but not ERK2. J Biol Chem 271: 24788-24793, 1996.
48. Sakuma H, Ikeda A, Oka S, Kozutsumi Y, Zanetta J-P and Kawasaki T: Molecular cloning and functional expression of a cDNA encoding a new member of mixed lineage protein kinase from human brain. J Biol Chem 272: 28622-28629, 1997.
49. Nagata K-I, Puls A, Futter C, Aspenstrom P, Schaefer E, Nakata T, Hirokawa N and Hall A: The MAPK kinase kinase kinase MLK2 co-localizes with activated JNK along microtubules and associates with kinesin subfamily motor KIF3. EMBO J 17: 149-158, 1998.

50. Hirai S-I, Noda K, Moriguchi T, Nishida E, Yamashita A, Deyama T, Fukuyama K and Ohno S: Differential activation of two JNK activators, MKK7 and SEK1, by MKN28-derived non-receptor serine/threonine kinase/mixed lineage kinase 2. J Biol Chem 273: 7406-7412, 1998.
51. Hirai S-I, Katoh M, Terada M, Kyriakis JM, Zon LI, Rana A, Avruch J and Ohno S: MST/MLK2, a member of the mixed lineage kinase family, directly phosphorylates and activates SEK1, an activator of c-Jun N-terminal kinase/stress-activated protein kinase. J Biol Chem 1997: 15167-15173, 1997.
52. Katz P, Whalen G and Kehrl JH: Differential expression of a novel protein kinase in human B lymphocytes. Preferential localization in the germinal center. J Biol Chem 269: 16802-16809, 1994.
53. Yuasa T, Ohno S, Kehrl JH and Kyriakis JM: Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH_2-terminal kinase (JNK) and p38. J Biol Chem 273: 22681-22692, 1998.
54. Ren M, Zeng J, Lemos-Chiarandini D, Rosenfeld M, Adesnik M and Sabatini DD: In its active form, the GTP-binding protein rab8 interacts with a stress-activated protein kinase. Proc Natl Acad Sci USA 93: 5151-5155, 1996.
55. Hu MC-T, Qiu WR, Wang X, Meyer CF and Tan T-H: Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. Genes Dev 10: 2251-2264, 1996.
56. Kiefer F, Tibbles LA, Anafi M, Janssen A, Zanke BW, Lassam N, Pawson T, Woodgett JR and Iscove NN: HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. EMBO J 15: 7013-7025, 1996.
57. Anafi M, Kiefer F, Gish GD, Mbamalu G, Iscove NN and Pawson T: SH2/SH3 adaptor proteins can link tyrosine kinases to a Ste20-related protein kinase, HPK1. J Biol Chem 272: 27804-27811, 1997.
58. Ling P, Yao Z, Meyer CF, Wang XS, Oehrl W, Feller SM and Tan T-H: Interaction of HPK1 with the adaptor proteins Crk and CrkL leads to the synergistic activation of c-Jun N-terminal kinase (JNK). Mol Cell Biol 19: 1359-1368, 1999.
59. Oehrl W, Kardinal C, Ruf S, Adermann K, Groffen J, Feng G-S, Blenis J, Tan T-H and Feller SM: The germinal center kinase (GCK)-related protein kinases HPK1 and KHS are candidates for highly selective signal transducers of Crk family adapter proteins. Oncogene 17: 1893-1902, 1998.
60. Ensenat D, Yao Z, Wang XS, Kori R, Zhou G, Lee SC and Tan T-H: A novel Src homology 3 domain-containing adaptor protein, HIP-55, that interacts with hematopoietic progenitor kinase 1. J Biol Chem 274: 33945-33950, 1999.
61. Zhou G, Lee SC, Yao Z and Tan T-H: Hematopoietic progenitor kinase 1 is a component of transforming growth factor ß-induced c-Jun N-terminal kinase signaling cascade. J Biol Chem 274: 13133-13138, 1999.
62. Tung RM and Blenis J: A novel human SPS1/STE20 homologue, KHS, activates Jun N-terminal kinase. Oncogene 14: 653-659, 1997.
63. Shi C-S and Kehrl JH: Activation of stress-activated protein kinase/c-Jun N-terminal kinase, but not NF-κB, by the tumor necrosis factor (TNF) receptor 1 through a TNF receptor-associated factor 2- and germinal center kinase related-dependent pathway. J Biol Chem 272: 32102-32107, 1997.
64. Shi C-S, Tuscano JM, Witte ON and Kehrl JH: GCKR links the *Bcr-Abl* oncogene and Ras to the stress-activated protein kinase pathway. Blood 93: 1338-1345, 1999.
65. Diener K, Wang XS, Chen C, Meyer CF, Bray J, Zukowski M, Tan T-H and Yao Z: Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase. Proc Natl Acad Sci USA 94: 9687-9692, 1997.
66. Yao Z, Zhou G, Wang XS, Brown A, Diener K, Gan H and Tan T-H: A novel mammalian STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. J Biol Chem 274: 2118-2125, 1999.
67. Su Y-C, Han J, Xu S, Cobb M and Skolnik EY: NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. EMBO J 16: 1279-1290, 1997.
68. Fu CA, Shen M, Huang BCB, Lasaga J, Payan DG and Luo Y: TNIK, a novel member of the germinal center kinase family that activates the c-Jun N-terminal kinase pathway and regulates the cytoskeleton. J Biol Chem 274: 30729-30737, 1999.
69. Herskowitz I: MAP kinase pathways in yeast: for mating and more. Cell 80: 187-197, 1995.
70. Whitmarsh AJ and Davis RJ: Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. Trends Biochem Sci 23: 481-485, 1998.
71. Yasuda J, Whitmarsh AJ, Vavanagh J, Sharma M and Davis RJ: The JIP group of mitogen-activated protein kinase scaffold proteins. Mol Cell Biol 19: 7245-7254, 1999.
72. Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J and Davis RJ: A mammalian scaffold complex that selectively mediates MAP kinase activation. Science 281: 1671-1674, 1998.
73. Ito M, Yoshioka K, Akechi M, Yamashita S, Takamatsu N, Sugiyama K, Hibi M, Nakabeppu Y, Shiba T and Yamamoto K-I: JSAP1, a novel Jun-N-terminal protein kinase (JNK)-binding protein that functions as a scaffold factor in the JNK signaling pathway. Mol Cell Biol 19: 7539-7548, 1999.
74. Xu S and Cobb MH: MEKK1 binds directly to the c-Jun N-terminal kinases/stress-activated protein kinases. J Biol Chem 272: 32056-32060, 1997.
75. Hunter T: Protein kinases and phosphatases: the Yin and Yang of protein phosphorylation and signaling. Cell 80: 225-236, 1995.
76. Nell BG and Tonks NK: Protein tyrosine phosphatases in signal transduction. Curr Opin Cell Biol 9: 193-204, 1997.
77. Franklin CC and Kraft AS: Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. J Biol Chem 272: 16917-16923, 1997.
78. Muda M, Theodosiou A, Rodrigues N, Boschert U, Camps M, Gillieron C, Davies K, Ashworth A and Arkinstall S: The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. J Biol Chem 271: 27205-27208, 1996.
79. Chu Y, Solski PA, Khosravi-Far R, Der CJ and Kelly K: The mitogen-activated protein kinase phosphorylates PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity *in vivo* toward the ERK2 *sevenmaker* mutation. J Biol Chem 271: 6497-6501, 1996.
80. Muda M, Boschert U, Smith A, Antonsson B, Gillieron C, Chabert C, Camps M, Martinou I, Ashworth A and Arkinstall S: Molecular cloning and functional characterization of a novel mitogen-activated protein kinase phosphatase, MKP-4. J Biol Chem 272: 5141-5151, 1997.
81. Tanoue T, Moriguchi T and Nishida E: Molecular cloning and characterization of a novel dual specific phosphatase, MKP-5. J Biol Chem 274: 19949-19956, 1999.
82. Ward Y, Gupta S, Jensen P, Wartmann M, Davis RJ and Kelly K: Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. Nature 367: 651-654, 1994.
83. Rohan PJ, Davis P, Moskaluk CA, Kearns M, Krutzsch H, Siebenlist U and Kelly K: PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. Science 259: 1763-1766, 1993.
84. Sun H, Charles CH, Lau LF and Tonks NK: MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase *in vivo*. Cell 75: 487-493, 1993.
85. Grumont RJ, Rasko JEJ, Strasser A and Gerondakis S: Activation of the mitogen-activated protein kinase pathway induces transcription of the PAC-1 phosphatase gene. Mol Cell Biol 16: 2913-2921, 1996.
86. Bokemeyer D, Sorokin A, Yan M, Ahn NG, Templeton DJ and Dunn MJ: Induction of mitogen-activated protein kinase phosphatase 1 by the stress-activated protein kinase signaling pathway but not extracellular signal-regulated kinase in fibroblast. J Biol Chem 271: 639-642, 1996.
87. Brondello J-M, Brunet A, Pouyssegur J and Mckenzie FR: The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the $p42/p44^{MAPK}$ cascade. J Biol Chem 272: 1368-1376, 1997.
88. Steller H: Mechanisms and genes of cellular suicide. Science 267: 1445-1449, 1995.
89. Thompson CB: Apoptosis in the pathogensis and treatment of disease. Science 267: 1456-1462, 1995.
90. Salvesen G and Dixit VM: Caspases: intracellular signaling by proteolysis. Cell 91: 443-446, 1997.
91. Kawakami Y, Miura T, Bissonnette R, Hata D, Khan W, Kitamura T, Maeda-Yamamoto M, Hartman S, Yao L, Alt FW and Kawakami T: Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. Proc Natl Acad Sci USA 94: 3938-3942, 1997.

92. Yu K, Ravera CP, Chen Y-NP and McMahon G: Regulation of Myc-dependent apoptosis by p53, c-Jun N-terminal kinase/ stress-activated protein kinases, and Mdm-2. Cell Growth Differ 8: 731-742, 1997.
93. Liu YF: Expression of polyglutamine-expanded huntingtin activates the SEK1-JNK pathway and induces apoptosis in a hippocampal neuronal cell line. J Biol Chem 273: 28873-28877, 1998.
94. Harkin DP, Bean JM, Miklos D, Song Y-H, Truong VB, Englert C, Christians FC, Ellisen LW, Maheswaran S, Oliner JD and Haber DA: Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. Cell 97: 575-586, 1999.
95. Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenberg ME: Opposing effects of ERK and JNK-p38 MAP kinase on apoptosis. Science 270: 1326-1331, 1995.
96. Chen Y-R, Wang X, Templeton D, Davis RJ and Tan T-H: The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and γ radiation: duration of JNK activation may determine cell death and proliferation. J Biol Chem 271: 31929-31936, 1996.
97. Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z and Kolesnick RN: Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 380: 75-79, 1996.
98. Zanke BW, Boudreau K, Rubie E, Winnett E, Tibbles LA, Zon L, Kyriakis J, Liu F-F and Woodgett JR: The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. Curr Biol 6: 606-613, 1996.
99. Sawai H, Okazaki T, Yamamoto H, Okano H, Takeda Y, Tashima M, Sawada H, Okuma M, Ishikura H, Umehara H and Domae N: Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells. J Biol Chem 270: 27326-27331, 1995.
100. Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R and Johnson EMJ: Altered gene expression in neurons during programmed cell death: identification of c-Jun as necessary for neuronal apoptosis. J Cell Biol 127: 1717-1727, 1994.
101. Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Vaniv M and Rubin LL: A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. Neuron 14: 927-939, 1995.
102. Behrens A, Sibilia M and Wagner EF: Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. Nat Genet 21: 326-329, 1999.
103. Liu Z-G, Hsu H, Goeddel DV and Karin M: Dissection of TNF receptor 1 effector function: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell 87: 565-576, 1996.
104. Natoli G, Costanzo A, Ianni A, Templeton DJ, Woodgett JR, Balsano C and Levero M: Activation of SAPK/JNK by TNF receptor 1 through a non-cytotoxic TRAF2-dependent pathway. Science 275: 200-203, 1997.
105. Lenczowski JM, Lourdes D, Eder AM, King LB, Zacharchuk CM and Ashwell JD: Lack of a role for Jun kinase and AP-1 in Fas-induced apoptosis. Mol Cell Biol 17: 170-181, 1997.
106. Goillot E, Raingeaud J, Ranger A, Tepper RI, Davis RJ, Harlow E and Sanchez I: Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway. Proc Natl Acad Sci USA 94: 3302-3307, 1997.
107. Brenner B, Koppenhoefer U, Weinstock C, Linderkamp O, Lang F and Gulbins E: Fas- or Ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/ p38 kinases and GADD153. J Biol Chem 272: 22173-22181, 1997.
108. Toyoshima F, Moriguchi T and Nishida E: Fas induces cytoplasmic apoptotic responses and activation of the MKK7-JNK/ SAPK and MKK6-p38 pathways independent of CPP32-like proteases. J Cell Biol 139: 1005-1015, 1997.
109. Chen Y-R, Meyer CF and Tan T-H: Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in γ radiation-induced apoptosis. J Biol Chem 271: 631-634, 1996.
110. Guo Y-L, Baysal K, Kang B, Yang L-J and Williamson JR: Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-α in rat mesangial cells. J Biol Chem 273: 4027-4034, 1998.
111. Guo Y-L, Kang B and Williamson JR: Inhibition of the expression of mitogen-activated protein phosphatase-1 potentiates apoptosis induced by tumor necrosis factor-α in rat mesangial cells. J Biol Chem 273: 10362-10366, 1998.
112. Qian M, Kralova J, Yu W, Bose HR, Dvorak M, Sanders BG and Kline K: c-Jun involvement in vitamin E succinate induced apoptosis of reticuloendotheliosis virus transformed avian lymphoid cells. Oncogene 15: 223-230, 1997.
113. Chen Y-R, Wang W, Kong A-NT and Tan T-H: Molecular mechanisms of c-Jun N-terminal kinase (JNK)-mediates apoptosis induced by anticarcinogenic isothiocyanates. J Biol Chem 273: 1769-1775, 1998.
114. Sanchez-Perez I, Murguia JR and Perona R: Cisplatin induces a persistent activation of JNK that is related to cell death. Oncogene 16: 533-540, 1998.
115. Lee L-F, Li G, Templeton DJ and Ting JP-Y: Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun N-terminal kinase (JNK/SAPK). J Biol Chem 273: 28253-28260, 1998.
116. Meriin AB, Gabai VL, Yaglom J, Shifrin VI and Sherman MY: Proteasome inhibitors activate stress kinases and induce Hsp72. Diverse effects on apoptosis. J Biol Chem 273: 6373-6379, 1998.
117. Luo Y, Umegaki H, Wang X, Abe R and Roth GS: Dopamine induces apoptosis through an oxidation-involved SAPK/JNK activation pathway. J Biol Chem 273: 3756-3764, 1998.
118. Faris M, Kokot N, Latinis K, Kasibhatla S, Green DR, Koretzky GA and Nel A: The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating fas ligand expression. J Immunol 160: 134-144, 1998.
119. Shan R, Price JO, Gaarda WA, Monia BP, Krantz SB and Zhao ZJ: Distinct roles of JNKs/p38 MAP kinase and ERKs in apoptosis and survival of HCD-57 cells induced by withdrawal or addirion of erythropoietin. Blood 94: 4067-4076, 1999.
120. Chen Y-R, Zhou G and Tan T-H: c-Jun N-terminal kinase mediates apoptotic signaling induced by N-(4-hydroxyphenyl) retinamide. Mol Pharmacol 56: 1271-1279, 1999.
121. Xu X, Heidenreich O, Kitajima I, McGuire K, Li Q, Su B and Nerenberg M: Constitutive activated JNK is associated with HTLV-1 mediated tumorigenesis. Oncogene 12: 135-142, 1996.
122. Galley Y, Hagens G, Glaser I, Davis W, Eichhorn M and Dobbelaere D: Jun N-terminal kinase is constitutively activated in T cells transformed by the intracellular parasite *Theileria parva*. Proc Natl Acad Sci USA 94: 5119-5124, 1997.
123. Antonyak MA, Moscatello DK and Wong AJ: Constitutive activation of c-Jun N-terminal kinase by a mutant epidermal growth factor receptor. J Biol Chem 273: 2817-2822, 1998.
124. Nagata Y and Todokoro K: Requirement of activation of JNK and p38 for environmental stress-induced erythroid differentiation and apoptosis and of inhibition of ERK for apoptosis. Blood 94: 853-863, 1999.
125. Gupta K, Kshirsagar S, Li W, Gui L, Ramakrishana S, Gupta P, Law PY and Hebbel RP: VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ ERK and SAPK/JNK signaling. Exp Cell Res 247: 495-504, 1999.
126. Sakata N, Patel HR, Terada N, Aruffo A, Johnson GL and Gelfand EW: Selective activation of c-Jun kinase mitogen-activated protein kinase by CD40 on human B cells. J Biol Chem 270: 30823-30828, 1995.
127. Berberich I, Shu G, Siebelt F, Woodgett JR, Kyriakis JM and Clark EA: Cross-linking CD40 on B cells preferentially induces stress-activated protein kinases rather than mitogen-activated protein kinases. EMBO J 15: 92-101, 1996.
128. Shafman TD, Saleem A, Kyriakis J, Weichselbaum R, Kharbanda S and Kufe DW: Defective induction of stress-activated protein kinase activity in Ataxia-Telangiectasia cells exposed to ionizing radiation. Cancer Res 55: 3242-3245, 1995.
129. Shafman T, Khanna KK, Kedar P, Spring K, Kozlov S, Yen T, Hobson K, Gatei M, Zhang N, Watters D, Egerton M, Shiloh Y, Kharbanda S, Kufe D and Lavin M: Interaction between ATM protein and c-Abl in response to DNA damage. Nature 387: 520-523, 1997.
130. Kharbanda S, Ren R, Pandey P, Shafman TD, Feller SM, Weichselbaum RR and Kufe DW: Activation of the c-Abl tyrosin kinase in the stress response to DNA-damaging agents. Nature 376: 785-788, 1995.
131. Kharbanda S, Pandey P, Ren R, Mayer B, Zon L and Kufe D: c-Abl activation regulates induction of the SEK1/stress-activated protein kinase pathway in the cellular response to 1-ß-D-arabinofuranosylcytosine. J Biol Chem 270: 30278-30281, 1995.

132. Park DS, Stefanis L, Irene Yan CY, Farinelli SE and Greene LA: Ordering the cell death pathway. J Biol Chem 271: 21898-21905, 1996.
133. Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, Pincus MR, Sardana M, Henderson CJ, Wolf CR, Davis RJ and Ronai Z: Regulation of JNK signaling by GSTp. EMBO J 18: 1321-1334, 1999.
134. Saitoh M, Nishitoh H, Fujii M, Takedo K, Tobiume K, Sawada Y, Kawabata M, Miyazono K and Ichijo H: Mammalian thioredoxin is a direct inhibitor of apoptosis signals-regulating kinase. EMBO J 17: 2596-2606, 1998.
135. Gotoh Y and Cooper J: Reactive oxygen species- and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-α signal transduction. J Biol Chem 273: 17477-17482, 1998.
136. Walton KM and Dixon JE: Protein tyrosine phosphatases. Annu Rev Biochem 62: 101-120, 1993.
137. Tonks NK, Diltz CD and Fischer EH: Purification of the major protein-tyrosine-phosphatases of human placenta. J Biol Chem 263: 6722-6730, 1988.
138. Jacobson MD: Reactive oxygen species and programmed cell death. Trends Biochem Sci 20: 83-86, 1996.
139. Cahill MA, Peter ME, Kischkel FC, Chinnaiyan AM, Dixit VM, Krammer PH and Nordheim A: CD95 (APO-1/Fas) induces activation of SAP kinases downstream of ICE-like proteases. Oncogene 13: 2087-2096, 1996.
140. Cardone M, Salvesen GS, Widmann C, Johnson G and Frisch SM: The regulation of anoikis: MEKK1 activation requires cleavage by caspases. Cell 90: 315-323, 1997.
141. Widmann C, Gerwins P, Johnson NL, Jarpe MB and Johnson GL: MEK kinase 1, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. Mol Cell Biol 18: 2416-2429, 1998.
142. Deak JC, Cross JV, Lewis M, Qian Y, Parrott LA, Distelhorst CW and Templeton DJ: Fas-mediated proteolytic activation and intracellular redistribution of the stress-signaling kinase MEKK1. Proc Natl Acad Sci USA 95: 5595-5600, 1998.
143. Rudel T and Bokoch GM: Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation PAK2. Science 276: 1571-1574, 1997.
144. Chen Y-R, Meyer CF, Ahmed B, Yao Z and Tan T-H: Caspase-mediated cleavage and functional changes of hematopoietic progenitor kinase 1 (HPK1). Oncogene 18: 7370-7377, 1999.
145. Yang X, Khosravi-Far R, Chang HY and Baltimore D: Daxx, a novel fas-binding protein that activates JNK and apoptosis. Cell 89: 1067-1076, 1997.
146. Chang HY, Nishitoh H, Yang X, Ichijo H and Baltimore D: Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adaptor protein Daxx. Science 281: 1860-1863, 1998.
147. Yeh W-C, de la Pompa JL, McCurrach ME, Shu H-B, Elia AJ, Shahinian A, Ng M, Wakeham A, Khoo W, Mitchell K, El-Deiry WS, Lowe SW, Goeddel DV and Mak TW: FADD, essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 279: 1954-1958, 1998.
148. Zhang J, Cado D, Chen A, Kabra N and Winoto A: Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature 392: 296-300, 1998.
149. Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T, Holtmann H, Lonai P and Wallach D: Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity 9: 267-276, 1998.
150. Michaelson JS, Bader D, Kuo F, Kozak C and Leder P: Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. Genes Dev 13: 1918-1923, 1999.
151. Seimiya H, Mashima T, Toha M and Tsuruo T: c-Jun N-terminal kinase-mediated activation of the interleukin-1ß converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis. J Biol Chem 272: 4631-4636, 1997.
152. Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A and Green DR: DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-κB and AP-1. Mol Cell 1: 543-551, 1998.
153. Le-Niculescu H, Bonfoco E, Kasuya Y, Claret F-X, Green DR and Karin M: Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. Mol Cell Biol 19: 751-763, 1999.
154. Fuchs S, Adler V, Pincus MR and Ronai Z: MEKK1/JNK signaling stablizes and activates p53. Proc Natl Acad Sci USA 95: 10541-10546, 1998.
155. Owen-Schaub L, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang W-W, Kruzel E and Radinsky R: Wild-type human p53 and a temperature-sensitive mutant induce Fas/Apo-1 expression. Mol Cell Biol 15: 3032-3040, 1995.
156. Muller M, Strand S, Hug H, Heinemann E-M, Walczak H, Hofmann WJ, Stremmel W, Krammer PH and Galle PR: Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (Apo-1/Fas) receptor/ligand system and involves activation of wild-type p53. J Clin Invest 99: 403-413, 1997.
157. Bennett M, Macdonald K, Chan S-W, Luzio JP, Simari R and Weissberg P: Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science 282: 290-293, 1998.
158. Chen Y-R and Tan T-H: Lack of correlation in JNK activation and p53-dependent Fas expression induced by apoptotic stimuli. Biochem Biophys Res Commun 256: 595-599, 1999.
159. Noguchi K, Kitanaka C, Yamana H, Kokubu A, Mochizuki T and Kuchino Y: Regulation of c-Myc through phophorylation at Ser-62 and Ser-71 by c-Jun N-terminal kinase. J Biol Chem 274: 32580-32587, 1999.
160. Yamamoto K, Ichijo H and Korsmeyer SJ: Bcl-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G2/M. Mol Cell Biol 19: 8469-8478, 1999.
161. Meikrantz W and Schlegel R: Apoptosis and the cell cycle. J Cell Biochem 58: 160-174, 1995.
162. Chang BS, Minn AJ, Muchmore SW, Fesik SW and Thompson CB: Identification of a novel regulatory domain in $Bcl\text{-}_{XL}$ and Bcl-2. EMBO J 16: 968-977, 1997.
163. Haldar S, Basu A and Croce CM: Serine-70 is one of the critical sites for drug-induced Bcl-2 phosphorylation in cancer cells. Cancer Res 58: 1609-1615, 1998.
164. Maundrell K, Antonsson B, Magnenat E, Camps M, Muda M, Chabert C, Gillieron C, Boschert U, Vial-Knecht E, Martinou J-C and Arkinstall S: Bcl-2 undergoes phophorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. J Biol Chem 272: 25238-25242, 1997.
165. Haldar S, Basu A and Croce CM: Bcl-2 is the guardian of microtubule integrity. Cancer Res 57: 229-233, 1997.
166. Blagosklonny M, Giannakakou P, El-Deiry W, Kingston DGI, Higgs PI, Neckers L and Fojo T: Raf-1/Bcl-2 phosphporylation: a step from microtubule damage to cell death. Cancer Res 57: 130-135, 1997.
167. Herr I: JNK/SAPK activity contributes to TRAIL-induced apoptosis. Cell Death Differ 6: 130-135, 1999.
168. Muhlenbeck F, Haas E, Schwenzer R, Schubert G, Grell M, Smith C, Scheurich P and Wajant H: TRAIL/Apo2L activates c-Jun N-terminal kinase (JNK) via caspase-dependent and caspase-independent pathways. J Biol Chem 273: 33091-33098, 1998.
169. Kharbanda S, Saleem A, Shafman T, Emoto Y, Taneja N, Rubin E, Weichselbaum R, Woodgett J, Avruch J, Kyriakis J and Kufe D: Ionizing radiation stimulates a Grb2-mediated association of the stress-activated protein kinase with phosphatidylinositol 3-kinase. J Biol Chem 270: 18871-18874, 1995.
170. Saleem A, Dattaa R, Yuan Z-M, Kharban da S and Kufe D: Involvement of stress-activated protein kinase in the cellular response to 1-ß-D-arabinofuranosylcytosine and other DNA-damaging agents. Cell Growth Differ 6: 1651-1658, 1995.
171. Wang T-H, Popp DM, Wang HS, Saitoh M, Mural JG, Henley DC, Ichijo H and Wimalasena J: Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and independent pathways in ovarian cancer cells. J Biol Chem 274: 8208-8216, 1999.
172. Zhang Y, Huang Y, Rishi AK, Sheikh MS, Shroot B, Reichert U, Dawson M, Poirer G and Fontana JA: Activation of the p38 and JNK/SAPK mitogenic-activated protein kinase pathways during apoptosis is mediated by a novel retinoid. Exp Cell Res 247: 233-240, 1999.
173. Lannuzel A, Barnier JV, Hery C, Huynh VT, Guibert B, Gry F, Vincent JD and Tardieu M: Human immunodifficiency virus type 1 and its coat protein gp120 induce apoptosis and activate JNK and ERK mitogen-activated protein kinases in human neurons. Ann Neurol 42: 847-856, 1997.

174. Westwick JK, Beilawska AE, Dbaibo G, Hannun YA and Brenner DA: Ceramide activates the stress-activated protein kinases. J Biol Chem 270: 22689-22692, 1995.
175. Skov S, Klausen P and Claesson MH: Ligation of major histocompatability complex (MHC) class I molecules on human T cells induces cell death through PI-3 kinase-induced c-Jun N-terminal kinase activity: a novel apoptotic pathway distinct from Fas-induced apoptosis. J Cell Biol 139: 1523-1531, 1997.
176. Yin T, Sandhu G, Wolfgang CD, Burrier A, Webb RL, Rigel DF, Hai T and Whelan J: Tissue-specific pattern of Stress kinase activation in ischemic/reperfused heart and kidney. J Biol Chem 272: 19943-19950, 1997.
177. Herdegen T, Claret FX, Kallunki T, Martin-Villalba A, Winter C, Hunter T and Karin M: Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinase after neuronal injury. J Neurosci 18: 5124-5135, 1998.
178. Zanke B, Lee C, Arab S and Tannock I: Death of tumor cells after intracellular acidification is dependent on stress-activated protein kinases (SAPK/JNK) pathway activation and cannot be inhibited by Bcl-2 expression ot interleukin 1 ß enzyme inhibition. Cancer Res 58: 2801-2808, 1998.
179. Moreno-Manzano V, Ishikawa Y, Lucio-Cazana J and Kitamura M: Suppression of apoptosis by all-trans-retinoic acid. Dual intervention in the c-Jun N-terminal kinase AP-1 pathway. J Biol Chem 274: 20251-20258, 1999.
180. Shtil AA, Mandlekar S, Yu R, Walter RJ, Hagen K, Tan T-H, Roninson IB and Kong A-NT: Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. Oncogene 18: 377-384, 1999.
181. Yu R, Jiao J-J, Duh J-L, Tan T-H and Kong A-NT: Phenyl isothiocyanate, a natural chemopreventive agent, activates c-Jun N-terminal kinaseI. Cancer Res 56: 2954-2959, 1996.
182. Yu R, Shtil AA, Tan T-H, Roninson IB, Kong A-NT: Adriamycin activates c-Jun N-terminal kinase in human leukemia cells: a relevance to apoptosis. Cancer Lett 107: 73-81, 1996.